

CHEMICAL, IMMUNOCHEMICAL AND SEROLOGICAL STUDIES
ON THE ABO, LEWIS AND P BLOOD GROUP SYSTEMS

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Lisbeth Messeter
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SAMMANFATTNING A new urinary A-active tetrasaccharide was isolated and characterized with chemical and serological methods. The excretion of blood group active oligosaccharides in urine after oral or intravenous administration of galactose and after starvation was studied and further proof was obtained that the intestine is the site of synthesis of these substances. By means of a new method for release of oligosaccharides from biological material, trifluoroacetolysis, the red cell content of some antigens within the P and ABO systems was confirmed and the usefulness of this method for studies of cell-bound carbohydrates was documented. Monoclonal antibodies for routine blood grouping were produced after immunization of mice with blood group substances, a synthetic glycoconjugate or human red cells. A large number of mouse-mouse hybridoma antibodies were obtained with anti-A, anti-B, anti-A,B and anti-Le ^b specificity. The ABO antibodies were tested in manual and automated routine blood grouping on a large number of samples. Some of the antibodies were shown to be superior to existing polyclonal testsera of human origin. Five agglutinating anti-Le ^b antibodies, one of them a potent anti-Le ^{BL} , were obtained. The anti-Le ^{BL} antibody was better than existing testsera of human origin and at least as good as commercial sera of animal origin. The specificity of this antibody was confirmed in immunochemical studies with a large number of well-characterized oligosaccharides and glycolipids.			
NYCKELORD Blood group active oligosaccharides; Urinary excretion; Biosynthesis; ABO, Lewis and P blood group systems; Galactose administration; Red cell membranes; Trifluoroacetolysis; Gas chromatography-Mass spectrometry; Monoclonal antibodies.			
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TILLÄMPNINGSGOMRÅDE Undersökningen leder till ökad förståelse av blodgruppsantigenens kemiska natur. De framtagna monoklonala antikropparna kommer att medföra förbättring av det blodgruppsserologiska arbetet.			
NYCKELORD Kolhydratkemi; Kolhydratbiosyntes; Immunkemi; Blodgruppsserologi; Monoklonala antikroppar.			
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by

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LUND 1983



ABBREVIATIONS USED

Fuc	<u>L</u> -fucose
Gal	<u>D</u> -galactose
GalNAc	<u>N</u> -acetyl- <u>D</u> -galactosamine
Glc	<u>D</u> - glucose
GlcNAc	<u>N</u> -acetyl- <u>D</u> -glucosamine
Cer	Ceramide
Lact-cer	Lactosyl ceramide

LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. Isolation and characterization of a blood group A-specific urinary tetrasaccharide.
C. Derappe, A. Lundblad, L. Messeter and S. Svensson.
FEBS letters 119 (1980) 177-180.
- II. Urinary excretion of oligosaccharides induced by galactose given orally or intravenously.
M.A. Chester, P. Hallgren, A. Lundblad and L. Messeter.
Eur. J. Biochem. 100 (1979) 385-392.
- III. Release of oligosaccharides from human erythrocyte membranes of different blood-group-P phenotypes by trifluoroacetolysis.
A. Lundblad, S. Svensson, B. Löw, L. Messeter and B. Cedergren.
Eur. J. Biochem. 104 (1980) 323-330.
- IV. Mouse monoclonal antibodies with anti-A, anti-B and anti-A,B specificity; superior to human polyclonal ABO reagents.
L. Messeter, T. Brodin, M.A. Chester, B. Löw and A. Lundblad.
Submitted for publication. Vox Sang.
- V. Immunochemical characterization of a monoclonal anti-Le^b blood grouping reagent.
L. Messeter, T. Brodin, M.A. Chester, K-A. Karlsson, D.A. Zopf and A. Lundblad.
Submitted for publication. Vox Sang.

INTRODUCTION

Blood group serology is essentially a study of simple phenomena, of which haemagglutination is by far the most important. The usefulness of blood transfusion in clinical medicine is well known, as is the necessity to avoid the ever present possibility of life-threatening blood group incompatibility. These reactions are caused by the interaction of an antigen on the erythrocyte surface with an antibody circulating in the blood plasma, and this presence or absence of haemagglutination when serum is mixed with erythrocytes from different individuals is the empirical basis of serology. The use of this simple technique, sometimes with certain modifications, and the meticulous observations made by many serologists throughout this century, has resulted in the elucidation of an impressive number of blood group systems and their antigens, which is a cornerstone of human genetics.

It is against the principles of science to merely accept the phenomenon of haemagglutination without elucidating the nature of the reaction. However, determining the structure of the antigens and antibodies involved has proved to be a gargantuan task. The complex area of antibody structure and diversity is beyond the scope of this thesis, which will concentrate on some blood group antigens.

The obvious source of blood group antigens is the erythrocyte, but it was soon found that the nature of the erythrocyte antigens was too complex and the amounts present too low to allow antigen purification and structural analysis with the methods currently available. Attention then turned to other possible sources of blood group substances, and using serological methods such as inhibition of haemagglutination and precipitation of antigen-antibody complexes it was in some cases possible to find rich sources of blood group substances in a form conducive to purification. Much useful information was obtained in this way before recent advances in separation techniques and methods of structural analysis made it possible to attack the erythrocyte membrane directly and discover its secrets.

That the structural information currently available on blood group antigens is almost exclusively limited to carbohydrates is no coincidence. This can be attributed to the retention of serological activity due to the relative stability of carbohydrate structures during the isolation and degradation of antigenic determinants from complex biological mixtures. The use of antibodies in structural studies is, however, not problem-free since most reagents are in fact polyclonal antisera and thus suffer from the disadvantage of being relatively uncharacterized and of having variable properties. The development of monoclonal antibodies has removed many of these anxieties and is becoming a very useful tool in studies on blood group antigens. Indeed, the accumulating knowledge of the structures of these antigens is currently being used in attempts to characterize the monoclonal antibody binding sites.

Despite impressive chemical and biochemical progress many problems remain. The most studied blood group system, the ABO system, is still not free from controversy and indeed, the chemical nature of the majority of the blood group antigens is still largely unknown.

Besides the obvious practical significance of blood groups it should be remembered that the intense study of these systems has been an invaluable model which has had a profound effect on the development of human genetics, structural carbohydrate chemistry and the whole field of complex carbohydrate metabolism.

HISTORICAL BACKGROUND

Agglutination reactions between red cells and serum from different individuals were first observed at the end of the last century, but it was the classical paper of Landsteiner (1) in 1901 which revealed that human blood could be divided into three distinct groups. Landsteiner realized the practical importance of the reactions between red cells and the "naturally occurring" haemagglutinins (antibodies) and his observations are the foundation of modern transfusion practice.

These initial studies also formed the basis for further research on the occurrence of other inherited blood group characters. Immunization of laboratory animals with human or monkey red cells resulted in the formation of agglutinating antibodies which after purification by extensive absorption were tested on a very large number of human blood samples. Certain specific reaction patterns were sometimes obtained. In this way, antigens within the MN, P and Rhesus blood group systems were detected.

A second phase in the detection and description of new blood group antigens started around 1945, when new methods became available to detect the so-called "incomplete" or "blocking" antibodies (now known to be IgG antibodies) that do not agglutinate saline-suspended red cells. These methods, used when investigating sera mainly from individuals who had received blood transfusions or who had been pregnant, led to the discovery of many new antibodies. After studying the reactions of these antibodies on blood cells from a very large number of individuals, frequently in families and different ethnic groups, the existence of new blood group antigens could be established.

With very few exceptions, blood group antigens are inherited characters, generally already present in foetal life, although in the very young the antigenic strength may be weaker than on adult red cells.

Today, more than 400 red cell antigens are known, frequently grouped into blood group systems (Table I lists some blood group systems; see also Race and Sanger (2)) and the year when the first antigen within the system was detected.

Table I.

Some blood group systems

1901 ABO	1945 Lutheran	1959 Kidd	1962 Scianna
1927 MNSs	1946 Kell	1955 Diego	1965 Dombrock
1927 P	1946 Lewis	1956 Cartwright	1965 Colton
1940 Rhesus	1948 Hh	1956 Ii	1967 Sid
	1950 Duffy	1969 Auberger	

This list does not include antigens still not assigned to a blood group system. The work described in this thesis is restricted to three of these systems, namely ABO, Lewis and P.

GENES AND ANTIGENS WITHIN THE ABO, LEWIS AND P SYSTEMS

I. The ABO system

In addition to Landsteiner's pioneering work, the discovery of a fourth blood group (3), the subsequent subdivision of A into A₁ and A₂ (4,5) and the postulation that the A and B genes are co-dominant whereas the O gene is recessive (6), led to the basic formulation of the ABO groups as they are known today (Table II).

Table II.

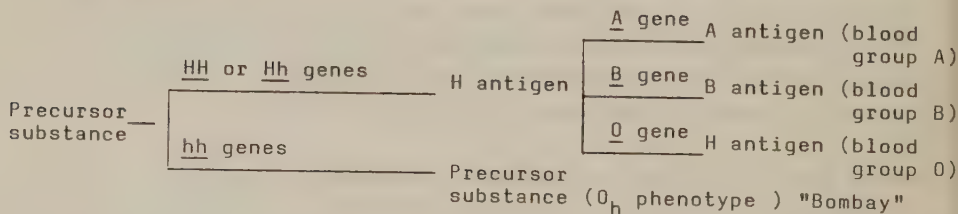
The ABO blood group system

Genotype	Red cell phenotype	Antibodies in serum
$\left. \begin{array}{l} \underline{A_1A_1} \\ \underline{A_1A_2} \\ \underline{A_1O} \end{array} \right\}$	A_1	Anti-B
$\left. \begin{array}{l} \underline{A_2A_2} \\ \underline{A_2O} \end{array} \right\}$	A_2	Anti-B
$\left. \begin{array}{l} \underline{B B} \\ \underline{B O} \end{array} \right\}$	B	Anti-A
$\underline{A_1B}$	A_1B	-
$\underline{A_2B}$	A_2B	-
$\underline{O O}$	O	Anti-A + anti-B

One outstanding characteristic of the ABO system is the regular occurrence of antibodies against the missing antigen(s).

The O gene is amorphous and what was hitherto thought to be the O antigen is the product of another gene, H, not at the ABO locus (7). This antigen can be converted to an A or B antigen by the enzyme products of the A or B genes (8). Individuals homozygous for the silent allele h cannot form ABH antigens and show the rare "Bombay" (O_h) phenotype (9). This concept is summarized in Figure 1.

Figure 1.

Genetic pathways for the biosynthesis of blood group ABH substances

The ABH antigens occur not only on human red cells and in plasma (10) but also on endothelial and epithelial (11) cells and have been recovered from tumour tissue (15,16). These antigens also occur in soluble form in secretions such as saliva, gastric juice and milk, and in urine (17-19). The presence of ABH substances in secretions is

dependent on yet another genetic system (20), the secretor genes (Se).

Individuals homozygous for the se allele have no ABH activity in their secretions, although the ABH activity of their red cells is not affected.

ABH-like substances are also found in other animal species (4,21) in micro-organisms and even in some plants (22-24).

II. The Lewis system

The Lewis system consists of two antigens (25,26), only one of which is usually present on an individual's erythrocytes. Despite this, the Lewis antigens are not the products of allelic genes. The presence or absence of a Lewis antigen is determined by the Le gene locus, but which of the two Lewis antigens appears is controlled by the same Hh and Se loci that influence the expression of the ABH groups (27). If at least one H and one Se gene are present, the Le^b antigen is expressed on red cells and both Le^a and Le^b antigens occur in saliva.

Three red cell phenotypes are possible (Table III). Antibodies against Lewis antigens are fairly common in human sera but not regularly occurring.

Table III.

The Lewis blood groups

Genotype	Red cell phenotype	Antigens on red cells	Antigens in secretions	Antibodies in serum
$\left. \begin{array}{l} \underline{SeSe} \text{ or } \underline{SeSe} \\ \underline{LeLe} \text{ or } \underline{LeLe} \end{array} \right\}$	$Le(a-b+)$	Le^b	Le^a, Le^b	-
<u>se</u> se, <u>Le</u> Le or <u>Le</u> le	$Le(a+b-)$	Le^a	Le^a	Anti- Le^b (rarely)
<u>Se</u> Se, <u>Se</u> se or <u>se</u> se; <u>le</u> le	$Le(a-b-)$	-	-	Anti- Le^a +anti- Le^b

The Lewis antigens can be demonstrated on red cells although they are not synthesized during erythropoiesis but are passively acquired from the plasma (28,29). These antigens also occur on endothelial and epithelial cells (30), in secretions (31-33) and in plasma (29). Lewis antigens have also been recovered from tumour tissue (34). Certain anthropoid apes show Lewis-like antigens (21) but so far they have not been demonstrated in lower species.

III. The P system

For a long time, the P system was considered to be a fairly simple two-allele system. The P_1 blood group antigen was detected in 1927 by means of antibodies obtained after immunization of rabbits with human red cells (35), but not until 1955 was it clear that other antigens also belong to this system (36). Even today the genetics of this system are not clear. Five phenotypes are now known (table IV). Antibodies against P_1 antigens are common, and occur regularly in those few individuals who lack all P antigens (see also Race and Sanger) (37)

Table IV.

The P blood group system

Phenotype	Red cell antigens	Antibodies in serum
P_1	P_1, P	-
P_2	P	Anti- P_1
P_1^k	P_1, P^k	Anti-P
P_2^k	P^k	Anti- P_1 + anti-P
$\bar{P}$	-	Anti- P_1 + anti-P + anti- P^k

P blood group antigens occur on red cells (38,39), on lymphocytes and fibroblasts (40), on epithelial cells (41,42) and in plasma (43). The P_1 antigenic determinant has been found in glycoproteins from sheep hydatid cyst fluid (44), and the P antigen has been found in other animal species (42,45,46).

THE STRUCTURE AND BIOSYNTHESIS OF ANTIGENIC DETERMINANTS

I. The ABO and Lewis antigens

At first, extraction of active material from erythrocyte membranes would seem most straightforward for determination of ABH structures. Early studies had shown that minute amounts of ABH-active material could be extracted with organic solvents (47,48) and that the active material probably consisted of lipid substances. However, the methods available at that time were not particularly sensitive and required large amounts of starting material. When it was found that ovarian cyst fluid contains large amounts of water-soluble blood group active glycoproteins (49), this material was used for most of the original structural determinations.

Early studies with simple sugars had shown that lectin-mediated agglutination of group A and O red cells could be inhibited by N-acetyl-D-galactosamine and L-fucose, respectively (50). Lectins were used since monosaccharides do not inhibit human antisera (51). Confirmation of the involvement of these two sugars, and the demonstration of the importance of D-galactose in the blood group B determinant, was possible by studying the inhibition of immuno-precipitation of purified blood group substances by these sugars (50). Similar conclusions were reached by studying the changes in blood group activity mediated by the action of specific glycosidases (52).

Chemical analysis of these blood group substances demonstrated the presence of the three sugars, L-fucose, D-galactose and N-acetyl-D-galactosamine, and in addition, N-acetyl-D-glucosamine, in proportions that did not vary significantly between individuals of different ABO group (53).

Mild hydrolysis of purified blood group substances from donors of different blood groups yielded a number of oligosaccharides characteristic for each group (54). By testing the ability of these and other oligosaccharides to inhibit haemagglutination by human anti-A and anti-B antisera, the structures of the A and B antigenic determinants were established (Table V).

Table V.

Structures of the A, B, H, Le^a and Le^b determinants

GalNAcα1-3Galβ1-3(or -4)GlcNAc...	A determinant (55,56.)
2	
1	
Fuca	
Galα1-3Galβ1-3(or -4)GlcNAc...	B determinant (55,57)
2	
1	
Fuca	
Galβ1-3(or -4)GlcNAc...	H determinant (56,58)
2	
1	
Fuca	
Galβ1-3GlcNAc...	Le ^a determinant (59)
4	
1	
Fuca	
Galβ1-3GlcNAc...	Le ^b determinant (60)
2	4
1	1
Fuca	Fuca

Surprisingly, the only difference between the A and B determinants is a substitution of a hydroxyl group (B) by an acetylated amino group (A). Using almost identical procedures, the H determinant was shown to be the common underlying structure (Table V). The structures of the Lewis antigenic determinants (Table V) were elucidated in a similar manner, using fragments isolated from cyst blood group substances and oligosaccharides isolated from human milk.

It is clear that the A, B, H and Lewis antigenic determinants are closely related, as would be expected from the known genetical and serological data. The link between the blood group genes and antigens was forged when it was postulated (61) and subsequently demonstrated (8) that the primary products of the active genes are glycosyltransferases capable of a stepwise biosynthesis of the carbohydrate antigenic determinants (62). It is noteworthy that the ABH determinants can be made on both type I and II chains but the Lewis determinants can only be synthesized on type I chains (Figure 2a). The mono- and difucosyl structures based on the type II chain (Figure 2b), analogous to the Lewis antigenic determinants, have no known antigenic activity.

This section gives a condensed summary of the extensive experimentation carried out over many years by serologists, chemists and enzymologists which has resulted in the understanding of the ABO and Lewis blood group systems. For a complete review, the reader is referred to a recent article by W.M. Watkins (63).

II. The P antigens

The elucidation of the structures of the antigens in the P blood group system has in many ways followed the same pattern as that observed for the ABO and Lewis systems. Inhibition of agglutination of P₁ erythrocytes by human anti-P₁ antibody using mono- and oligosaccharides indicated that α -D-galactose played an important role (64). Sheep hydatid cyst fluid was found to contain a glycoprotein which exhibits P₁ activity (64). This activity could be destroyed by a specific α -4-galactosidase (65) and mild hydrolysis of the hydatid substance yielded two oligosaccharides with the structures Gal α 1-4Gal and Gal α 1-4Gal β 1-4GlcNAc (65). The latter structure was shown to have strong P₁ activity (66) and both oligosaccharides showed P^k activity (67).

Figure 2a.

Biochemical modification of Gal β 1-3GlcNAc... (Type I) structures

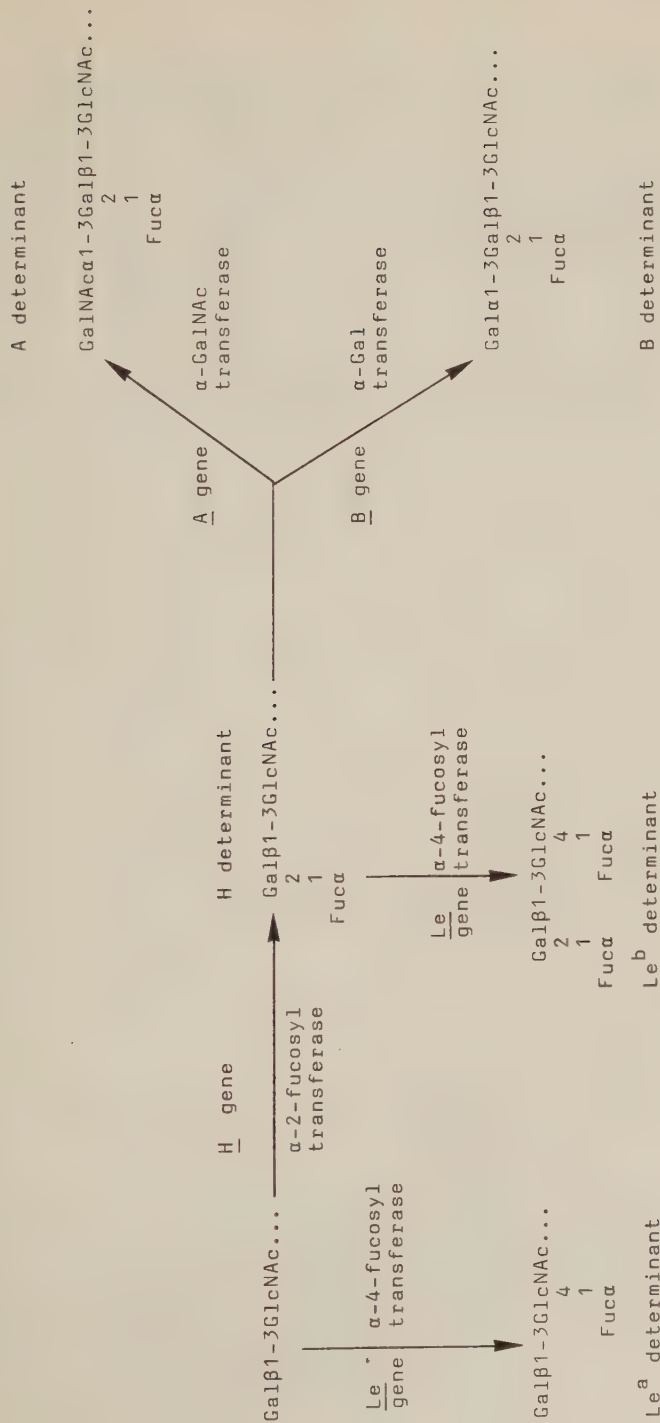
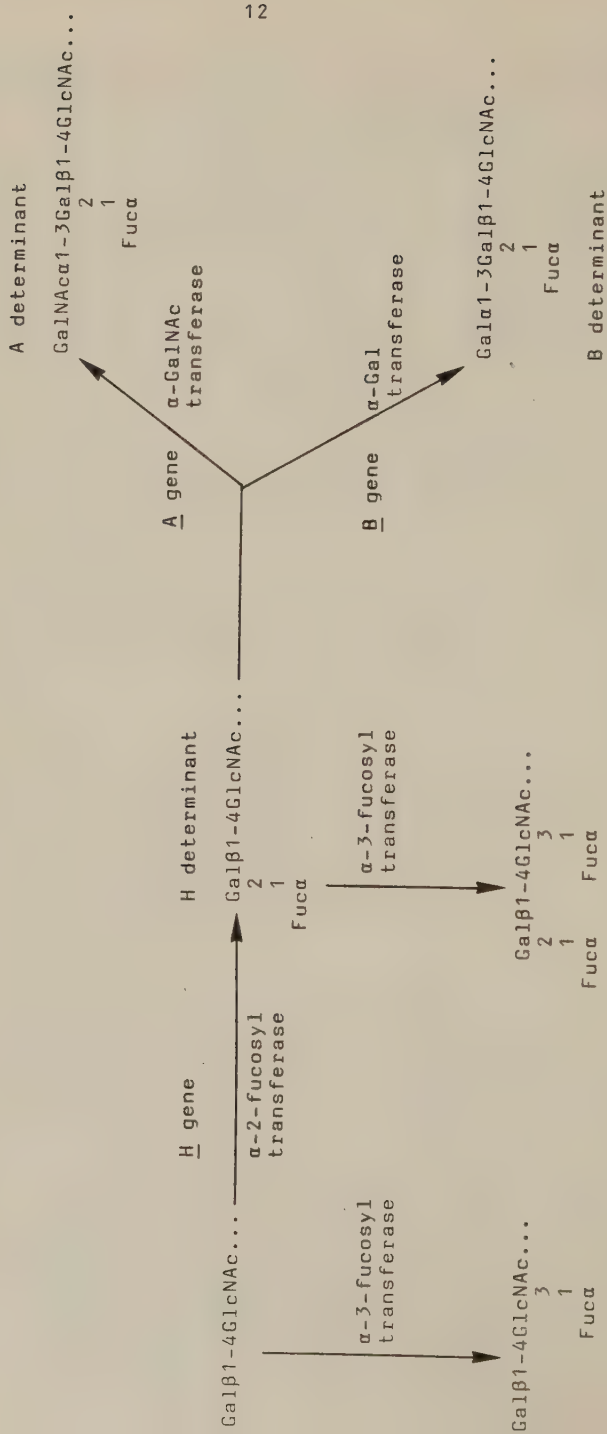


Figure 2b.

Biochemical modification of Gal β 1-4GlcNAc... (Type II) structures



The red cell's P and P^k antigens were shown to be the well-known erythrocyte glycolipids globoside and trihexosyl ceramide respectively (68, 69). Extension of this work (39, 70) indicated that the P₁ antigen is a ceramide pentasaccharide containing the same terminal trisaccharide unit as that isolated from hydatid cysts (see above). The structures of these antigens are shown in Table VI.

Table VI.

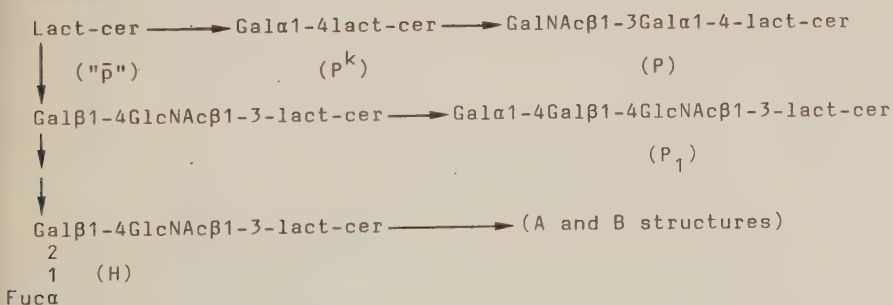
Glycosphingolipid antigens of the P blood group system

Antigen	Structure	Trivial name
P ₁	Gal α 1-4Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc-Cer	P ₁ -glycolipid
P	GalNAc β 1-3Gal α 1-4Gal β 1-4Glc-Cer	Globoside
P ^k	Gal α 1-4Gal β 1-4Glc-Cer	Trihexosyl ceramide
($\bar{P}$)	Gal β 1-4Glc-Cer	Lactosyl ceramide

The biosynthetic routes for the formation of the various antigens in the P system are shown in figure 3. The close relationship to the ABH antigens is also shown.

Figure 3.

Formation of erythrocyte glycolipids involved in the P blood group system



From the figure it can be seen that the ultimate formation of the P^k and P₁ antigens depends on the action of an α -1-4-galactosyltransferase and the formation of the P antigen requires in addition a β -1-3-N-acetylgalactosaminyltransferase.

Several genetic models for the P system have been proposed (39,70-73). The product of the $\underline{P}$ gene is almost certainly the β -N-acetylgalactosaminyltransferase converting the P^k antigen to the P antigen (38,74). The most probable explanation for the occurrence of the P_1 , P^k and $\bar{p}$ phenotypes is the presence of three alleles $\underline{P}_1$, $\underline{P}^k$ and $\underline{p}$ (73). The $\underline{P}_1$ and $\underline{P}^k$ genes would code for different α -1-4-galactosyltransferases, the product of the $\underline{P}_1$ gene being of broader specificity, thus being able to synthesize the P^k and P_1 structures, whereas the product of the $\underline{P}^k$ gene would have a stricter acceptor specificity and only be able to synthesize the P^k antigen. The $\underline{p}$ gene would be amorphous. Similar theories have been postulated in which the different acceptor specificities of the α -galactosyltransferases are explained by the presence of a modifier gene, presumably at yet another locus (75).

THE PRESENT INVESTIGATION

I. Studies on urinary blood group active oligosaccharides

High molecular weight glycoproteins with ABH activity are excreted in the urine of ABH secretors (19,76). This material, which is poly-disperse, has not been characterized in any great detail but is probably very similar to the soluble blood group substances isolated from gastric juice and ovarian cyst mucins.

Urine is a rich source of oligosaccharides with ABH activity (77), and the structures of the compounds that have been isolated in greatest yield are shown in table VII.

Table VII.

Some important urinary oligosaccharides with A, B and H activity

A-active structures		B-active structures	
GalNAc α 1-3Gal		Gal α 1-3Gal	
2		2	
1		1	
Fuca		Fuca	
GalNAc α 1-3Gal β 1-4Glc		Gal α 1-3Gal β 1-4Glc	
23		23	
11		11	
FucaFuca		FucaFuca	
H-active structures			
Gal			
2			
1			
Fuca			
Gal β 1-4Glc			
23			
11			
FucaFuca			

The non-reducing terminals of these oligosaccharides are identical to those of ABH active glycolipids from erythrocyte membranes or ABH active soluble blood group substances from various mucins. They can be looked upon as free ABH haptens.

The blood group activity of the A and B difucosylated pentasaccharides was studied by inhibition of precipitation using human polyclonal antisera, and was found to correspond well with the activity of difucosylated oligosaccharides obtained after partial hydrolysis of A and B soluble blood group substances (78). The A and B trisaccharides from urine were more active than the difucosyl compounds (79).

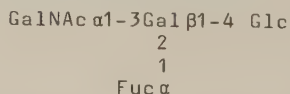
It was also observed that the excretion of the trisaccharides was strictly dependent on the administration of galactose (79), either in free form or glycosidically bound, as in lactose. The excretion rate of larger blood group active oligosaccharides has also been reported to be dependent on the administration of galactose or lactose (80).

Experiments on a blood group B Rhesus monkey (81) which was given (^{14}C)-labelled galactose either per os or via a mesenteric vein, strongly indicated that the intestine is the site of synthesis of the urinary B trisaccharide.

The site of biosynthesis of the larger blood group active oligosaccharides is not known, but the intestine is probably implicated and lactose might function as the substrate. The limited resorption of oligosaccharides across the intestine might explain why the urinary excretion of these oligosaccharides is relatively independent of diet. Individuals who display considerably increased excretion of A and B pentasaccharides after lactose ingestion might have a lower intestinal lactase activity than those who have minimally increased excretion.

a). A new A-active tetrasaccharide (Paper I)

In the course of routine examination of the urinary oligosaccharide excretion pattern by gas-liquid chromatography-mass spectrometry (GLC-MS) a new component was discovered in some but not all urines of A secretors. This new compound was isolated by gel chromatography and paper chromatography. The structure:



was elucidated by sugar analysis, methylation analysis, optical rotation studies and inhibition of the precipitin reaction.

This compound has more blood group A activity than the A tri- and pentasaccharides and compares well with the most active fragments from soluble blood group A substances. Biosynthesis of this tetrasaccharide is assumed to take place in the intestine since ingestion of both galactose and lactose resulted in a somewhat increased excretion rate.

The A-active tetrasaccharide is more suitable than the tri- or pentasaccharide for chemical coupling to protein to make an immunogen, or after coupling to some insoluble material for use in affinity chromatography.

b). Further studies on oligosaccharide formation induced by galactose administration (Paper II)

In order to obtain further evidence for the contention that the A and B trisaccharides are biosynthesized in the intestine, galactose was administered both orally and intravenously to 18 secretors of various ABO groups, and the presence of the blood group active oligosaccharides in plasma and urine was determined by GLC-MS analysis.

Intravenous injection gave rise to varying amounts of oligosaccharide excretion (3-59 per cent of the amount formed after oral administration to the same individual). This variation is probably due to individual differences in utilizing the injected galactose. Any galactose reaching the intestinal epithelial cells via the circulation can presumably also be used as a substrate in the formation of these oligosaccharides. The results obtained give further support for the hypothesis that the intestine is the main site of biosynthesis of these oligosaccharides. The B trisaccharide was also identified in plasma in one B secretor individual after oral galactose administration and this observation excludes a renal or urogenital tract origin of these oligosaccharides. The total amount of oligosaccharide formed is variable and probably depends on several factors, such as intestinal passage rate and efficiency of absorption.

II. Studies on erythrocyte membrane-linked oligosaccharides (Paper III)

Trifluoroacetolysis (TFA) is a method recently introduced into carbohydrate chemistry (82) for use in structural investigation of complex carbohydrates (83). This technique also allows the release of intact oligosaccharides from glycolipids and glycoproteins (82,84).

In order to evaluate the usefulness of TFA for the release of membrane-bound oligosaccharides, erythrocytes of blood group P₁, P^k and $\bar{p}$ phenotypes were chosen for a model study. The P antigens of the red cell membrane are glycolipids and their structures have been described (see Table VI).

The erythrocyte membranes were degraded and completely solubilized by TFA. Oligosaccharide chains were released as their pertrifluoroacetylated derivatives which were subsequently de-O and de-N-trifluoroacetylated, reduced, N-acetylated, permethylated and analysed by GLC-MS.

The limitations of the GLC technique preclude the analysis of oligosaccharides containing more than four to five sugars, especially if hexosamines are present. Consequently, detection of the hexosamine-containing pentasaccharide which constitutes the carbohydrate part of the P₁ glycolipid would not be expected, nor was it found in the present study. However, the P antigen (globoside), which is also present on P₁ red cells, yielded the expected tetrasaccharide. Similarly, the P^k antigen (ceramide trihexoside) present on P₁^k cells yielded a trisaccharide. This trisaccharide was also found in small amounts in P₁ red cell extracts, indicating the incomplete biosynthesis of the P antigen from the P^k antigen. This finding could also explain the absence of anti-P^k antibody in the serum of all individuals other than those of the $\bar{p}$ phenotype.

Erythrocytes of the $\bar{p}$ phenotype lack all P antigens and are assumed to compensate for this deficiency (particularly of globoside) with an increased amount of lactosyl ceramide, which was also confirmed in this study. The B antigen was present on the cells studied and the B trisaccharide was detected in each case after TFA.

At least 50 other unidentified oligosaccharides were obtained and detected by GLC-MS but these could not be identified due to the limited amount of material available.

It was concluded that TFA is a useful method for releasing oligosaccharides from membranes and obtaining detailed information of cell surface carbohydrates.

III. Monoclonal antibodies against blood group antigens (Papers IV and V)

The use of monoclonal antibodies produced by the hybridoma technique (85) has enabled dramatic advances to be made in the biological sciences. It can now be predicted that monoclonal antibodies will replace conventional polyclonal antisera in many applications, including blood group serology. The aim of the present study was to optimize conditions for the production of agglutinating antibodies and to evaluate their usefulness in routine ABO and Lewis grouping.

The ABO and Lewis antigenic determinants occur as glycolipids, glycoproteins and as free oligosaccharides. A- and B-active immunogens in the form of whole cells, erythrocyte "ghosts" and purified glycoproteins from ovarian cyst fluid were tested, and good agglutinating antibodies of anti-A, anti-B and anti-A,B specificities were obtained. Whereas anti-B antibodies were obtained using erythrocytes or glycoprotein immunogens, and anti-A,B antibodies were obtained using A₂ or A₂B red cells, there was a marked reluctance of A erythrocytes to elicit useful anti-A antibodies. All anti-A antibodies that could be used to detect A subgroups and weak variants were raised against blood group A-active glycoproteins. The best anti-Le^b antibodies were raised against a synthetic glycoprotein antigen rather than glycoproteins from cyst material or erythrocytes.

It was found that the best antibodies were obtained when spleen cells from hyperimmunized mice were used for fusion. If soluble substances were used as immunogens, adoption of a final high-dose booster schedule resulted in formation of the most potent antibodies.

The useful clones obtained were grown both in cell cultures and intraperitoneally in Balb/c mice to produce ascites. However, the antibody titre in ascitic fluid was generally very low, probably depending on partial degradation of the labile IgM molecules (86). Due to this low titre, non-specific reactions caused by the mice's own immunoglobulins could not be avoided (87). Hybridoma supernatant fluid from the clones chosen was in all instances potent enough to be used without concentration.

Two potent anti-A, two anti-B and one anti-A,B antibody were selected for a detailed clinical evaluation. They all proved to be superior to the best commercial antibodies presently available, both in manual and automated blood grouping. The superior quality of the monoclonal reagents was most evident when A₂B red cells and weak ABO variants were tested. The avidity of these antibodies was also excellent. Such high avidity is important if the antibodies are to be used in grouping of whole blood samples. The titre of the antibodies was also impressive, the most potent anti-A having a higher titre than the WHO International Reference Serum.

The addition of sodium chloride to the supernatants caused a dramatic increase of the reaction rate of the monoclonal ABO antibodies over a wide range of concentrations. Other salts could also induce this effect. However, salt addition has had no effect on the reaction of the anti-Le^b antibodies so far tested.

One potent anti-Le^b antibody was obtained that showed the characteristics of an anti-Le^{bl} antibody, giving strong reactions with Le(a-b+) red cells of all ABO groups. This antibody was tested on a large number of samples and the reactions were compared to those obtained using commercial polyclonal sera of human and goat origin. The monoclonal anti-Le^b antibody was consistently better than the human polyclonal reagents, and at least as good as the goat antisera.

The specificity of this monoclonal antibody was further elucidated by ELISA and RIA using a large number of oligosaccharides and glycolipids. Binding to a number of Le^b-active structures was observed. The antibody also bound other difucosyl structures, but these structures are not present on erythrocytes.

GENERAL SUMMARY

A new blood group A active tetrasaccharide was isolated from the urine of a normal A₁ secretor individual. Conventional gel and paper chromatographic methods were used to isolate the oligosaccharide, and its structure was determined by sugar and methylation analysis using gas chromatography and mass spectrometry. The serological activity, determined in precipitation inhibition experiments, was found to be stronger than that of urinary A tri- and pentasaccharide.

The urinary excretion of blood group active di-, tri- and pentasaccharides was studied in 18 healthy secretor individuals of different ABH group after starvation, after oral ingestion of galactose, lactose or sucrose, and after intravenous injection of galactose. Furthermore, the plasma contents of B trisaccharide was determined in a B secretor person after oral ingestion of galactose. The excretion of di- and trisaccharides in all 18 individuals was shown to be largely dependent upon oral ingestion. Although considerable individual variation was observed the amounts excreted were between two and 34 times higher after oral ingestion than after intravenous injection of galactose. An interesting observation was that the ratio of excreted A trisaccharide/B trisaccharide was so much higher in A₁B than A₂B individuals as to permit a distinction between these two blood groups in urine. A pentasaccharide found in the same urines was shown not to be dependent on diet. These studies therefore further substantiate the assumption that intestinal cells are the site of synthesis of blood group active urinary di- and trisaccharides.

P and B blood group structures were isolated from human red cell membranes by trifluoroacetylolysis, a new method for release of the carbohydrate moiety of glycolipids and glycoproteins. The antigenic determinants P and P^k, and their alleged precursor $\bar{p}$, together with the B trisaccharide were identified by gas chromatography-mass spectrometry of their permethylated alditols. This method is therefore suitable for isolation of carbohydrate chains, particularly from glycolipids, in biological material.

The hybridoma technique was used to produce mouse-mouse monoclonal antibodies against ABH and Le^b antigens. Blood group substances, a synthetic glycoconjugate or human red cells were used for immunization of the mice and a large number of haemagglutinating antibodies were obtained. Two anti-A, two anti-B and one anti-Le^b antibody were at least as good as or superior to existing polyclonal reagents of human or animal origin, and also superior to existing monoclonal reagents of the same specificities. Furthermore, for the first time, monoclonal anti-A,B antibodies were obtained, also of excellent quality. The antibodies were tested in routine manual and automated blood grouping and their quality was further confirmed.

The specificity of the haemagglutinating anti-Le^b antibody, which was of anti-Le^{bl} type, was confirmed in several RIA and ELISA test systems. The binding to Le^b structures was confirmed in binding and inhibition tests, using a large number of natural oligosaccharides and glycolipids with and without Le^b activity.

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ISOLATION AND CHARACTERIZATION OF A BLOOD GROUP A-SPECIFIC URINARY TETRASACCHARIDE

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1. Introduction

Blood group A-, B- and H-active tetra- en penta-saccharides have been isolated from urine of starved ABH secretors [1,2]. Ingestion of free galactose or a glycoside of galactose (lactose) induces the formation and excretion of blood group specific di- and tri-saccharides [3,4] and small amounts of hexa- and heptasaccharides [4]. The serological activities of some A- and B-specific oligosaccharides have been studied and compared with those of oligosaccharides from soluble blood group A and B substances [3,5]. When urine from individuals of different ABO blood group and secretor status was analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS) for the content of various oligosaccharides, a new component was discovered in some, but not in all, urines of blood group A secretors. We now report the isolation, characterization and serological characterization of this new component.

2. Experimental

Urine produced during 6 h following 12 h starvation was collected from 10 healthy individuals of different ABO blood group and secretor status, after fasting or oral ingestion of an aqueous solution of galactose (0.35 g/kg body wt) or lactose (0.70 g/kg body wt). Water was allowed ad libitum. (20 l) was

also collected from a blood group A secretor without any dietary restrictions. All urines were stored at -20°C until required. The 6 h urine portions were deionized by passage through a mixed-bed ion-exchange resin. To 20 ml of the desalted urine 200 µg isomaltotetraose were added as internal standard and the sample was reduced and permethylated. The permethylated oligosaccharides were analyzed by GLC-MS [6].

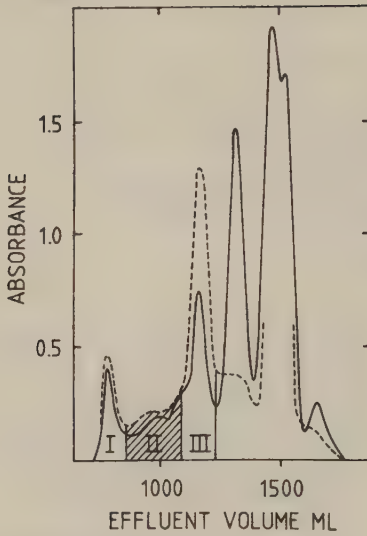
Isolation of the A-active tetrasaccharide was carried out using gel chromatography on Sephadex G-15 (for experimental details see legend to fig.1), and preparative paper chromatography was done on Whatman no. 3 papers using the developing systems (a) ethyl acetate/acetic acid/water 3/1/1 (by vol.) and (b) ethyl acetate/pyridine/water 10/4/3 (by vol.). Colorimetric methods were used for the determination of total hexose [7] and deoxyhexose [8]. The structure of the isolated oligosaccharide was deduced from results obtained from optical rotation, sugar and methylation analyses [1,2] and determination of serological activity [3,5,9]. The instrumentation and experimental conditions have been detailed in [10].

3. Results

Urine (20 l) from an A secretor was desalted, concentrated (rotatory evaporation) and fractionated by gel chromatography (fig.1a). The tetra-pentasaccharide region (II) was pooled as indicated, concentrated and further fractionated by preparative paper chromatography (fig.1b). Two main fractions were obtained (II₁, II₂). The latter had a mobility identical to urine A-pentasaccharide (for structure see table 1). Fraction II₁ was run preparatively also in solvent system b

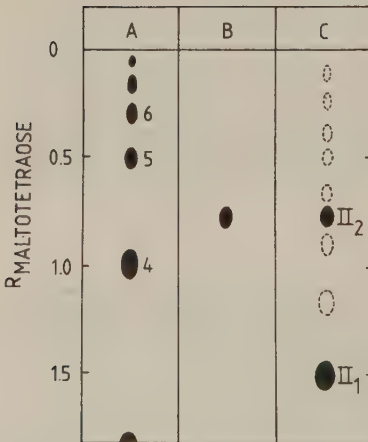
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a

b



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Table 1
Blood group A-active oligosaccharides

Trivial name (abbreviation)	Structure	Ref
A trisaccharide (A tri)	GalNAc(1 ₃)Gal a ₁ Fuc	(3)
A tetrasaccharide (A tetra)	GalNAc(1 ₃)Gal(1 ₅)Glc a ₁ Fuc	
A pentasaccharide (A penta)	GalNAc(1 ₃)Gal(1 ₅)Glc a ₂ a ₃ Fuc Fuc	(1)
A heptasaccharide (A hepta)	GalNAc(1 ₃)Gal(1 ₅)GlcNAc(1 ₅)Gal a ₂ a ₄ a ₆ Fuc Fuc Fuc	(4)
MSS AR ₀ O-52	GalNAc(1 ₃)Gal(1 ₅)GlcNAc(1 ₅)-R a ₂ a ₁ Fuc	(11)

and was thereafter found homogeneous in 5 additional solvent systems. The yield was 2.8 mg/urine.

Purified fraction II₁ ($[\alpha]_D^{20} + 52.5^\circ$) yielded on sugar analysis D-glucose, D-galactose, D-fucose, and N-acetyl-D-galactosamine in the relative molar proportions 1.0:0.9:0.9:1.1. These sugars accounted for >85% of the material. Small amounts of D-mannose were also present. By methylation analysis, the following partially methylated sugar derivatives were identified in about equimolar proportions: 2,3,4-tri-O-methyl-L-fucitol, 1,2,3,5,6-penta-O-methyl-D-glucitol-1-d, and 4,6-di-O-methyl-D-galactose. Two derivatives of hexosamine were found: 2-acetamido-2-deoxy-3,4,6-tri-O-methyl-D-galactitol; and 2-(N-methyl)-acetamido-2-deoxy-3,4,6-tri-O-methyl-D-galactitol.

The blood group A activity of compound II₁ was determined (fig.2). The activity was found to be almost as high as the most active oligosaccharide

Fig.1a. Gel chromatographic distribution of hexose- and deoxyhexose(fucose)-containing material in urine from a blood group A secretor. Concentrated urine (20 ml) was applied. The fractionation was performed on a Sephadex G-15 column (5 x 130 cm; V₀ 800 ml) elution rate, 70 ml/h; eluent, distilled water. (—) Total hexose; (---) fucose.

Fig.1b. Fractionation by paper chromatography in solvent (a). (A) Partial hydrolysate of starch: 4, maltotetraose; 5, maltopentaose; etc. (B) Urine A pentasaccharide. (C) Gel chromatographic fraction II₁.

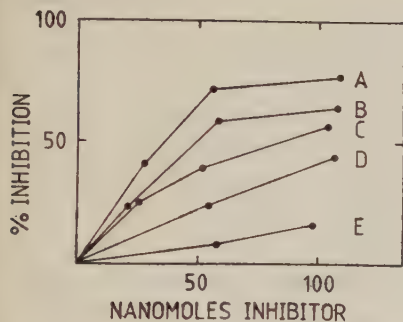


Fig. 2. Inhibition of precipitin reaction by different A-active oligosaccharides. Increasing amounts of oligosaccharide was added to 0.2 ml anti-A serum (ortho) and 12.2 μ g human A substance in 375 μ l a total vol. 0.15 M NaCl. (A) MSS AR₁ 0.52. (B) Urine A tetrasaccharide. (C) Urine A trisaccharide. (D) Urine A pentasaccharide. (E) Urine A heptasaccharide.

(MSS AR₁ 0.52) isolated from soluble blood group A substance and more active than any of the previously isolated A-active urinary oligosaccharides (table 1).

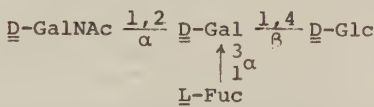
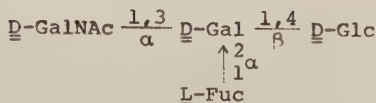
The results obtained are compatible with the structure given for urine A tetrasaccharide (table 1, fig. 3).

Quantitation of the urine A tetrasaccharide as well as of the urine A trisaccharide was done by GLC-MS.

The mass spectrum and primary fragmentation of reduced and permethylated A tetrasaccharide are given in fig. 3. Results from quantitation are seen in table 2. The A tetrasaccharide was present only in one out of four A secretors studied. The excretion rate increased slightly after ingestion of galactose or lactose but not to the same extent as is the case for the A trisaccharide.

4. Discussion

The data from sugar and methylation analyses of urine A tetrasaccharide are compatible with two different structures:



The high blood group A activity found makes the second structure highly unlikely, since the structure

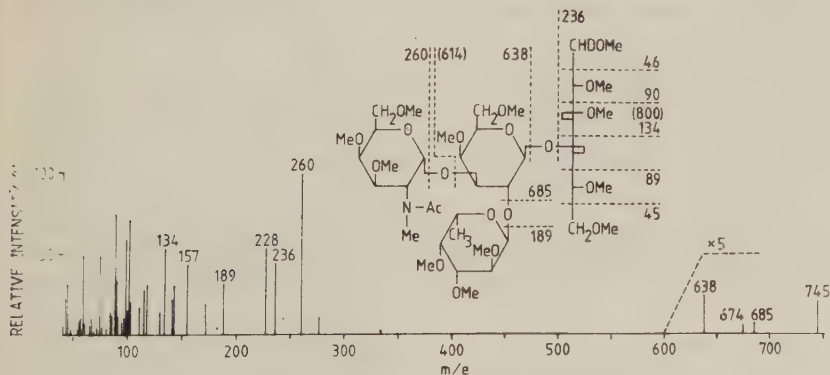


Fig. 3. Mass spectrum and some important primary fragments of urine A tetrasaccharide.

Table 2
Excretion rate^a of 'A tri' and 'A tetra' in urine of four blood group A individuals^b

Subject	Secretor status	Subgroup of A	Diet	'A tri' (mg/6 h)	'A tetra' (mg/6 h)
A.E.	+	A ₁	Starved	0	0.5
A.E.	+	A ₁	Oral galactose	74	2.0
A.E.	+	A ₁	Oral lactose	37	1.2
A.M.	+	A ₁	Oral lactose	49	0
A.L.	+	A ₁	Oral lactose	130	0
G.L.	-	A ₂	Oral lactose	0	0

^a Identification based on identical retention times and mass spectra as compared to those of authentic samples

^b 'A tri' and 'A tetra' were both absent in urines from individuals of other ABO blood groups

of the blood group A hapten is so well established that results from serological tests can serve as a structural proof. The quantitation data reveal a slight dependence in excretion rate on oral galactose or lactose intake, and in this respect 'A tetra' behaves similarly as 'A penta' but is completely different from 'A tri' which is absent in urine of starved A secretor urine but becomes the predominant oligosaccharide in normal urine after galactose or lactose ingestion.

The biosynthetic site for 'A tetra' as well as 'A penta' is unknown whereas the intestine is the most probable place for biosynthesis of the 'A tri', by analogy with what was shown for B-trisaccharide [12,13].

Analogous compounds might be expected to occur in urine of B and O(H) secretors, but have so far not been discovered with the following exceptions. In O(H) secretors the analogous compound would be 2'-fucosyllactose. This compound, which is a characteristic milk oligosaccharide has been found in urine of pregnant and lactating women [10] and is also present in urine of newborns receiving human milk (unpublished).

The reason why 'A tetra' is not found in all A secretors is not known although individual variability in the activity of different fucosyltransferases is a possibility.

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Urinary Excretion of Oligosaccharides Induced by Galactose Given Orally or Intravenously

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The effect of oral administration of galactose, lactose, and sucrose and intravenous injection of galactose on the urinary excretion of blood-group-active oligosaccharides has been studied. Galactose given either as the free sugar, a glycoside (lactose) or a constituent of normal diet was an absolute requirement for the formation and excretion of A-trisaccharide, B-trisaccharide and 2'-fucosylgalactose in blood group A, B and O(H) secretors, respectively. Great individual variation was seen in the amounts of galactose-dependent oligosaccharides excreted. Injection of galactose resulted in excretion of 3–59% of the amount of oligosaccharide formed after oral administration to the same individual. The mean ratio A-trisaccharide/B-trisaccharide was 2.7 in four blood-group-A₁B secretors and 0.22 in three A₂B secretors and can thus serve as a parameter for chemical differentiation between the two blood groups. The excretion of larger blood-group-active oligosaccharides, including the A-pentasaccharide, the B-pentasaccharide and lactodifucotetraose, that are normal components in urine from, respectively, starved A, B, and H secretors, was about the same after oral administration of galactose or lactose. The B-trisaccharide was the only oligosaccharide detected in plasma after oral galactose administration to a blood-group-B secretor individual. The concentration was 0.38 mg/l of plasma.

Blood-group-active tetrasaccharides and pentasaccharides have been isolated from the urine of starved blood-group A, B or H secretors [1–4]. Normal food intake or lactose diet induced an excretion of additional blood-group-active disaccharides or trisaccharides in these urines [5]. The oligosaccharides formed by A, B and H secretors are shown in Fig. 1. Strecker et al. [6] reported that both galactose and lactose induced urinary excretion of the A-active trisaccharide, and that the excretion of the A-pentasaccharide [1,4] decreased as a result of galactose diet and increased dramatically as a result of lactose diet. The dependence of the excretion of these oligosaccharides on diet suggests that they are products of biosynthesis and may be made in the intestinal cells. Experiments with a Rhesus monkey in which [¹⁴C]-galactose was administered both orally and via an intestinal vein, strongly indicated that the intestine is the site of biosynthesis [7]. In order to obtain further information about the biosynthesis of these oligo-

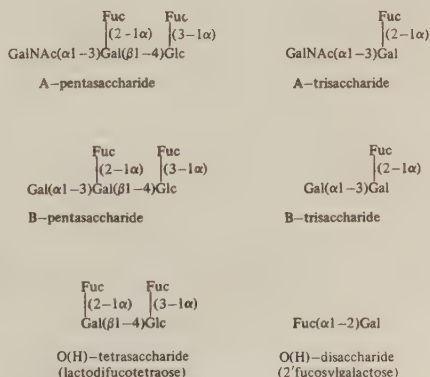


Fig. 1. Structures of six urinary blood-group-active oligosaccharides. Fuc, L-fucose; GalNAc, N-acetyl-D-galactosamine; Gal, D-galactose; Glc, D-glucose. All monosaccharides are in pyranoside form

saccharides, we have studied the effect of orally and intravenously administered galactose and orally administered lactose in nineteen individuals of different ABO blood groups.

EXPERIMENTAL PROCEDURE

Materials

Urine produced during 6 h following 12 h of starvation was collected from a healthy 25-year-old male, blood-group-A₁ Le(a-b+) secretor. Four other 6-h portions of urine were collected from the same individual following 12 h of starvation and oral administration of 25 g of galactose, 50 g of lactose, 50 g of sucrose, or intravenous administration of 21 g (350 mg/kg of body weight) of galactose [8]. Finally one 6-h portion of urine was collected without any dietary restrictions. Portions of urine (6 h) were also collected from secretors of different ABO blood groups following 12 h of starvation and intravenous or oral administration of 350 mg galactose/kg body weight *per os* (18 individuals) and intravenously (4 individuals).

Citrate-plasma (210 ml) was obtained from a healthy 25-year-old female, blood-group-B Le(a-b+) secretor. The plasma was collected 90 min after administration of 350 mg of galactose/kg body weight.

Analytical Methods

Ultrafiltration of filtered urine samples was performed at 4°C with Visking 23/32 dialysis tubing (Union Carb. Corp., Chicago, Ill.) and a negative pressure of 660 mm Hg [9]. Ultrafiltration of plasma was achieved with an Amicon ultrafiltration unit using a PM 30 membrane. Gel chromatography was carried out using Sephadex G-25 (fine) columns (10 × 105 cm, void volume 2700 ml; or 2.9 × 130 cm, void volume 400 ml) and a Sephadex G-15 column (5 × 130 cm, void volume 1000 ml). Whole urine and fractions obtained after gel chromatography were deionized by passage through columns packed with AG 50W-X8 (200–400 mesh, H⁺) and AG 3-X4A (100–200 mesh, OH⁻) (Bio-Rad Labs, Richmond, Calif.). Whatman No. 3 papers were used for descending paper chromatography in ethyl acetate/acetic acid/water (3/1/1, v/v/v). Papers were stained with a silver dip reagent [10]. Colorimetric methods were used for determining total hexose [11] and 6-deoxyhexose (fucose) [12] in fractions eluted from gel chromatography columns, ion exchangers, and preparative paper chromatograms. Sugar analyses were performed by gas-liquid chromatography [13] and mass spectrometry [14].

Methylation of reduced (NaB⁺H₄) oligosaccharides was carried out as described elsewhere [15,16]. Permethylated oligosaccharides were separated as their alditol derivatives on a Perkin-Elmer 3920 gas chromatograph equipped with a glass capillary column (25 m × 0.25 mm), wall-coated with SE-30 (LKB, Stockholm, Sweden). The separations of disaccharides were performed at 215°C and of trisaccharides using a linear temperature programme at 280–320°C (1°C/min). Sucrose, lactose, and 2'-fucosylgalactose were determined using melibiose (100 µg) as an internal standard and the A- and B-trisaccharide using maltotriose (100 µg) as an internal standard. Linear response was observed within a wide range (50–700 µg) for all the oligosaccharides. The response factors were as follows: lactose (*k* = 1.00), sucrose (*k* = 0.80), 2'-fucosylgalactose (*k* = 0.67), A-trisaccharide (*k* = 0.79), and B-trisaccharide (*k* = 0.99).

For gas-liquid chromatography/mass spectrometry an SE-30 column was connected to the ion source of a Varian MAT 311A combined gas-liquid chromatography/mass spectrometry instrument. The mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 1 mA, and an ion source temperature of 120°C. The data were processed by an on-line computer system (Spectrosystem 100, Varian MAT).

RESULTS

Gel Chromatographic Separation of Urinary Carbohydrate-Containing Material

The six urinary ultrafiltrates from the healthy male A₁ Le(a-b+) individual were concentrated tenfold and fractionated on a Sephadex G-25 column. Eluted fractions were analyzed for total hexose and 6-deoxyhexose (fucose) (Fig. 2). The characteristic A-secretor fucose pattern was seen in the 'starved' sample (Fig. 2A), with the A-pentasaccharide peak in region IV, and a disaccharide and a monosaccharide peak in regions VI and VII, respectively [1]. The additional trisaccharide peak in region V occurred in the "normal" sample (Fig. 2F) and became a predominant peak after oral administration of galactose or lactose (Fig. 2B and C). Peaks of approximately equal size were observed regardless of whether the galactose was given as the free sugar or as a glycoside in lactose. Sucrose did not give rise to any trisaccharide peak but gave a high peak of total hexose in the disaccharide region (Fig. 2D). Galactose given intravenously also induced a significant increase in fucose excretion in the trisaccharide region and furthermore a peak of total hexose in the disaccharide region was considerably increased (Fig. 2E). A slight increase of fucose-containing material in region III was seen

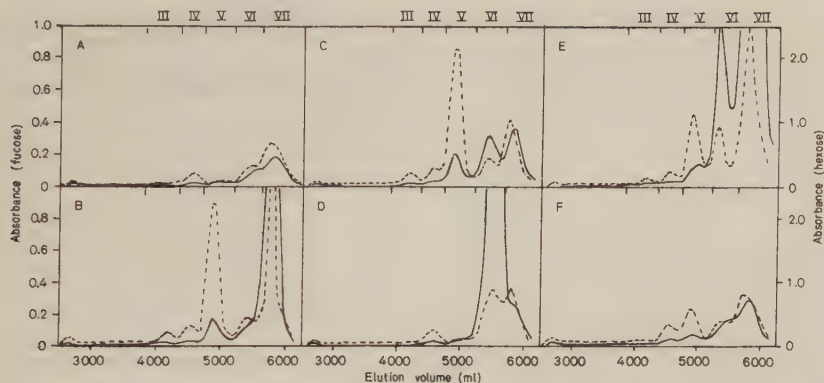


Fig. 2. Gel chromatographic profiles of 6-h urine samples from an A-secretor following (A) 12-h starvation, (B) 25 g galactose orally, (C) 21 g galactose intravenously, (D) 21 g galactose orally, (E) 21 g galactose intravenously, (F) no starvation, normal diet. Concentrated urine samples were applied to a column of Sephadex G-25 (10 × 105 cm, void volume 2700 ml) and eluted with distilled water. Eluted fractions (48 ml) were analyzed for total hexose (—) and 6-deoxyhexose (----). Material in regions III to VI, as indicated with vertical lines, was pooled for further analysis.

which, in each case, was roughly proportional to the increase in trisaccharide.

Quantitation of Disaccharides and Trisaccharides in Regions V and VI

The material obtained after gel chromatography was pooled into fractions III–VI as indicated (Fig. 2). Internal standards were added to each of fractions V and VI and the mixtures were subsequently methylated and analyzed by gas-liquid chromatography/mass spectrometry. The identification of lactose, sucrose, and the three blood-group-active oligosaccharides, was based on identical retention times and mass spectra as compared to those of authentic samples. The mass spectra of lactose and sucrose have been published previously [17,18]. The retention time of the A-trisaccharide was 1.48 relative to maltotriose and its mass spectrum and some important primary fragments are given in Fig. 3A. The amount of lactose and sucrose in fraction VI and A-trisaccharide in fraction V was quantitated by gas-liquid chromatography (Table 1).

Significant amounts of lactose and sucrose were excreted only when these sugars were ingested. After starvation, negligible amounts of A-trisaccharide were excreted, but oral administration of either galactose or lactose resulted in substantial excretion of the A-trisaccharide which was at least double that obtained when galactose was administered intravenously. The high hexose excretion in the disaccharide region after galactose injection consisted of a large number

of different galactose-containing disaccharides present as contaminants in the galactose solution injected.

Quantitation of Larger Oligosaccharides in Regions III and IV

Pooled fractions from regions III and IV were chromatographed on paper as described in Methods. Region IV from all experiments was identical showing only one main component which was identified as the A-pentasaccharide described previously [1,4]. Region III was resolved into two or three components (Fig. 4). Each urine sample contained the same compounds (III:1 and III:2) but compound III:3 was absent from the urines obtained after starvation, and oral administration of sucrose. The A-pentasaccharide and the three subfractions from region III were eluted from the paper and quantitated by total hexose and fucose determinations. The amount of A-pentasaccharide was approximately the same in all experiments (1.3–1.9 mg/6 h), but the amounts of the compounds in region III were considerably less (<0.5 mg/6 h).

Quantitation of the A-Trisaccharide, the B-Trisaccharide, and 2'-Fucosylgalactose in Urine from 18 Individuals of Different ABO Blood Groups after Oral or Intravenous Administration of Galactose

Ultrafiltered urine samples (20 ml) obtained after intravenous administration of galactose were concentrated by rotatory evaporation, then fractionated

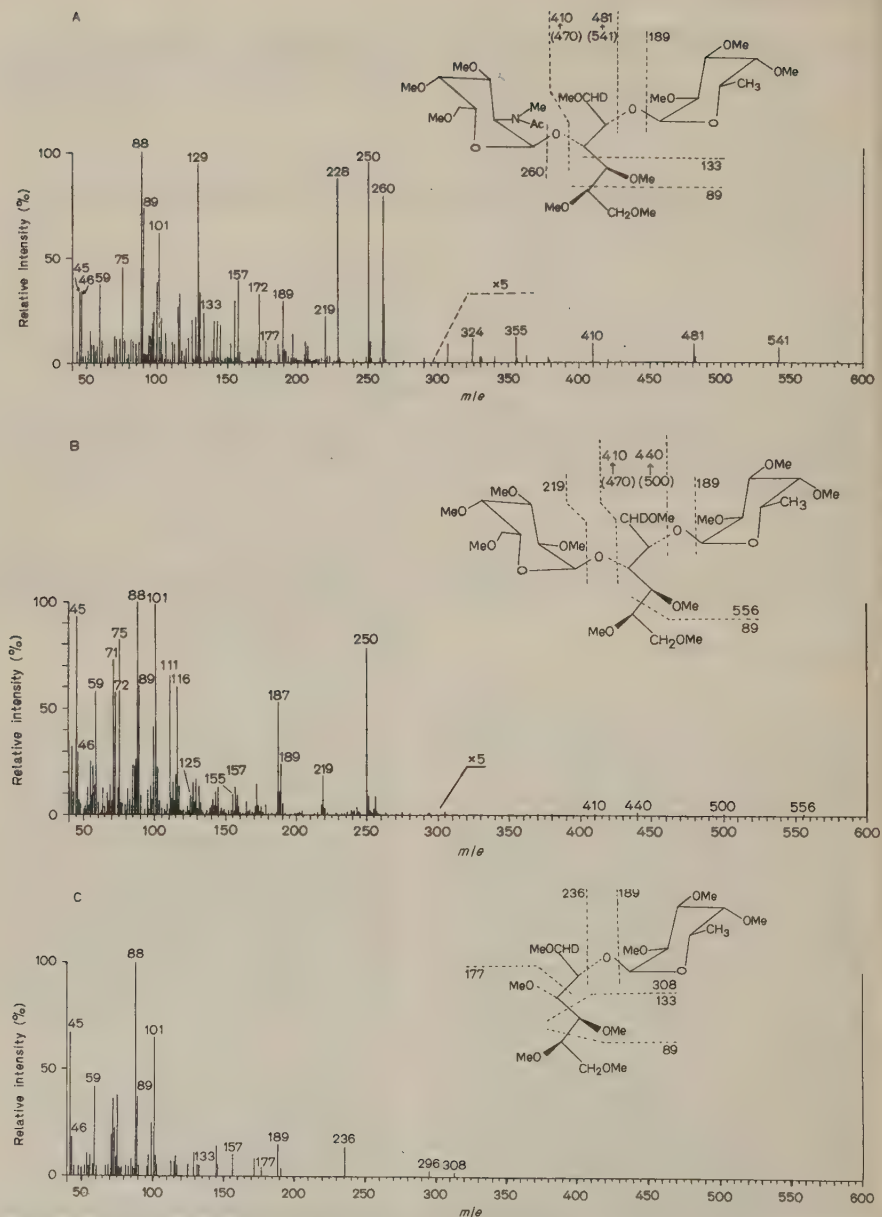


Fig. 3. Mass spectrum and some important primary fragments of (A) the A-trisaccharide, (B) the B-trisaccharide, and (C) 2-fucosylgalactose. Me, methyl; Ac, acetyl

Table 1. Rate of excretion of lactose, sucrose, and the A-trisaccharide by a blood-group-A₁ individual under various conditions
n.d., not determined

Treatment	Excretion in 6 h		
	lactose	sucrose	A-trisaccharide
	mg		
Starved	0.6	0	0
Oral galactose	0.5	0	20.4
Oral lactose	15.1	0	22.6
Oral sucrose	1.5	42.3	0.05
Intravenous galactose	n.d.	n.d.	9.4
Normal	2.6	0	7.4

on a Sephadex G-15 column. Eluted fractions were analyzed for total hexose and 6-deoxyhexose (fucose), and the material in the disaccharide fraction from O(H) secretors and in the trisaccharide fraction from A₂, B and A₁B secretors was pooled, deionized and concentrated by rotatory evaporation. Other ultrafiltered urine samples (20 ml) were deionized. To all samples 100 µg of the appropriate standard oligosaccharide was added and the sample was then reduced (NaB²H₄) and permethylated. All permethylated oligosaccharides were analyzed by gas-liquid chromatography/mass spectrometry as described above. The mass spectra and some important primary fragments from the B-trisaccharide and 2'-fucosylgalactose are given in Fig. 3B and C. The content of the oligosaccharides in urine was quantitated by gas-liquid chromatography (Table 2). The gas chromatograms obtained with one A₁B and one A₂B secretor after oral administration of galactose are seen in Fig. 5.

Determination of the B-Trisaccharide in Plasma after Oral Administration of Galactose

Galactose (350 mg/kg of body weight) was given orally to a female, blood-group-B Le (a-b+) secretor previously starved for 12 h. One unit (450 ml) of blood was collected 90 min later. The citrate-plasma from this blood sample (210 ml) was ultrafiltered and the ultrafiltrate concentrated tenfold by rotatory evaporation, then fractionated on a Sephadex G-25 column. Eluted fractions were analysed for total hexose and 6-deoxyhexose (Fig. 6). A large peak occurred in the monosaccharide region and some carbohydrate material eluted at and immediately after the void volume. In addition a small peak was observed in the trisaccharide region, which was pooled as indicated, reduced, permethylated, and analyzed by gas-liquid

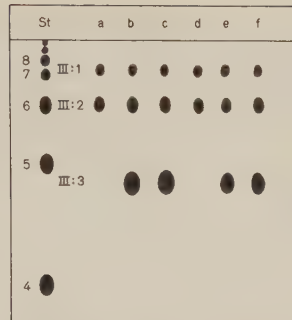


Fig. 4. Paper chromatographic separation of material from region III after gel chromatography of urine from a blood-group A₁-secretor individual. (a) Starved, (b) oral galactose, (c) oral lactose, (d) oral sucrose, (e) intravenous galactose, (f) normal diet. Separation was performed in ethyl acetate/acetic acid/water (3/1/1, v/v/v) for 10 days. A partial hydrolysate of starch (St) was used as reference; 4, maltotetraose; 5, maltopentaose, etc.

chromatography/mass spectrometry. One single component with retention time and mass spectrum identical with those of the B-trisaccharide was found. The concentration in the original plasma sample of this oligosaccharide was 0.38 mg/l of plasma.

DISCUSSION

Normal urine contains a large number of different low-molecular-weight carbohydrate compounds, particularly monosaccharides to trisaccharides. Established methods for the analysis of these compounds are time-consuming and do not necessarily allow good separation and recovery of individual components. Gas-liquid chromatography/mass spectrometry has proved to be an ideal method for rapid and accurate determinations of different oligosaccharides in complex biological materials [16,19] and was therefore used in the present study. Ultrafiltered deionized urine was reduced and permethylated directly in most cases. The method allows quantitative determination of sugars up to approximately tetrasaccharide size due to the lack of suitable volatile derivatives of the larger compounds. Colorimetric estimation of these larger oligosaccharides was carried out after gel chromatography and preparative paper chromatography. Intravenous injection of galactose resulted in the excretion of a large number of mainly disaccharide contaminants derived from the galactose solution used for injection. Gel chromatography removed most of these contaminants and was therefore included in all analyses of urines from patients injected with galactose.

Table 2. Quantitative determination of A-trisaccharide, B-trisaccharide and H-disaccharide in urines of 18 secretors of different ABO blood groups after oral (p.o.) or intravenous (i.v.) administration of galactose
Tri, trisaccharide; Di, disaccharide

Subject	ABO blood group	Excretion in 6 h						A-Tri/B-Tri	
		A-Tri		B-Tri		H-Di		p.o.	i.v.
		p.o.	i.v.	p.o.	i.v.	p.o.	i.v.		
mg									
A.L.	A ₁	102.9							
B.B.	A ₁	46.0							
S.M.	A ₁	18.1							
E.H.	A ₂	17.7	5.1						
H.B.W.	A ₂	13.4							
G.E.	B			34.0					
H.I.	B			19.7	11.8				
S.S.	B			46.1					
K.Ö.	A ₁ B	17.7		8.0				2.2	
E.A.	A ₁ B	35.7	8.8	11.0	2.1			3.2	4.2
S.T.M.	A ₁ B	23.8		9.7				2.5	
B.A.	A ₁ B	20.0		6.6				3.0	
G.H.	A ₂ B	5.0		20.8				0.24	
S.P.	A ₂ B	2.7		14.4				0.19	
C.F.	A ₂ B	6.5		26.1				0.25	
I.J.	O					12.4	0.36		
S.B.S.	O					14.1			
K.B.C.	O					17.1			

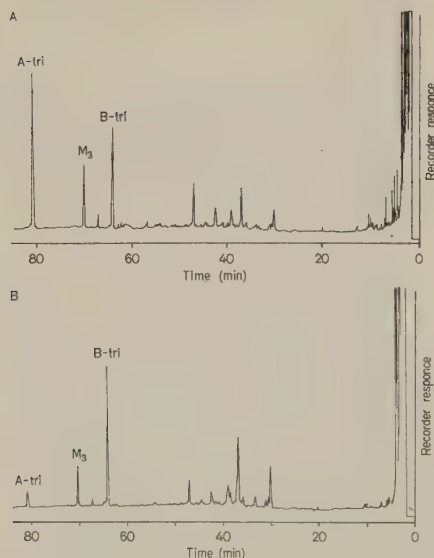


Fig. 5. Gas chromatogram obtained after injection of a reduced and permethylated sample of deionized (A) A₁B and (B) A₂B secretor urine collected after oral galactose administration. A-tri, A-trisaccharide; B-tri, B-trisaccharide; M₃, maltotriose

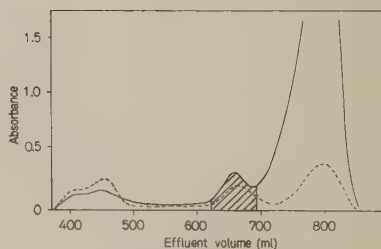


Fig. 6. Gel chromatography (Sephadex G-25; 2.9 × 130 cm, void volume 400 ml) of a concentrated plasma ultrafiltrate obtained from a blood-group-B-secretor woman after ingestion of galactose. Eluted fractions were analyzed for total hexose (—) and 6-deoxyhexose (fucose) (----). The trisaccharide region was pooled as indicated

The retention times and mass spectra of authentic samples of lactose, sucrose, 2'-fucosylgalactose, A-trisaccharide, and B-trisaccharide were obtained to identify these compounds in the individual urine samples.

The mass spectra obtained for lactose and sucrose agreed with previously published results [17,18]. The mass spectrum of the permethylated alditol of the A-trisaccharide (Fig. 3A) gave the following fragments

in the A-series: aA_1 (m/e 260), aA_2 (m/e 228), bA_1 (m/e 189), bA_2 (m/e 157), bA_3 (m/e 125), the following fragment of the J-series: $bAlaJ_1$ (m/e 541), and the fragments $bAla$ (m/e 481) and $aAlb$ (m/e 410). From these fragments it can be deduced that a deoxyhexose residue and acetamidodeoxyhexose residue are both linked to a hexitol. The fragmentation of the hexitol shows that it is substituted in the 2 and the 3 position. Similarly the fragments obtained from the B-trisaccharide (Fig. 3B) showed a hexose and a deoxyhexose residue both linked to a hexitol substituted in the 2 and 3 position. Fragments from 2'-fucosylgalactose (Fig. 3C) proved the deoxyhexose-(1-2)-hexitol structure.

Small amounts of lactose and sucrose were excreted when these sugars were administered orally (Table 1). Otherwise insignificant amounts were found in the urine. The ingestion of galactose, either as the free sugar or a glycoside (lactose), or as a constituent of normal diet resulted in an excretion of the A-trisaccharide.

The study of 18 secretors of different ABO blood groups given galactose orally (Table 2) showed that they all excreted the corresponding blood-group-specific disaccharide or trisaccharide. Within a particular blood group, the excretion of these oligosaccharides is dependent on the intestinal passage rate and the rate of absorption. However, the relative amounts of blood group oligosaccharide excreted in A_1 , A_2 , A_1B , A_2B , and B individuals compares favourably with the relative number of antigenic sites on red blood cells from the same groups [20]. Calculating the ratio between the amounts of the A-trisaccharide and B-trisaccharide excreted gave a parameter that clearly distinguishes between the two AB blood groups.

Previous experiments using a blood group B-secretor Rhesus monkey [7] demonstrated that a [^{14}C]-galactose-labelled B-trisaccharide was biosynthesized when [^{14}C]galactose was given orally but not when it was injected in the mesenteric vein. In the present work, injection of galactose into an upper limb resulted in the excretion of significant amounts of the blood-group-active trisaccharide. The considerable individual variation in the ratio of oligosaccharide excreted after oral or intravenous administration probably depends on the proportion of the blood pumped to various organs, and the efficiency of excretion of galactose through the kidneys, of uptake into the liver, and of mediated permeation into the intestine from the blood stream. Once the galactose has been transported into the intestinal epithelial cells the same process can take place which results in the urinary excretion of the blood-group-active disaccharides and trisaccharides.

The detection of the B-trisaccharide in plasma after oral galactose administration makes it very

unlikely that the kidney and the urogenital tract are the sites for biosynthesis of this oligosaccharide. No other disaccharides, trisaccharides or tetrasaccharides were found in plasma showing that if these are present their concentration is less than 50 $\mu g/l$ plasma, and a more sensitive analytical system would then have to be employed.

In each urine sample obtained from the A_1 Le(a-b+) secretor individual the amount of A-pentasaccharide was about the same (1.3–1.9 mg/6 h), demonstrating that the excretion rate is independent of the different experimental conditions used. This is in contrast to the results of Strecker et al. [6], who reported the excretion of 40 mg/6 h of A-pentasaccharide after ingestion of 40 g lactose, a difference we are unable to explain.

The larger oligosaccharides, III:1 and III:2 (Fig. 4), were also present in approximately equal amounts in each experiment (<0.5 mg/6 h). The low yield precluded characterization, although compound III:2 co-chromatographed with a sample of the A-active heptasaccharide, VIIA, obtained from Dr G. Strecker, who found an excretion of 10–25 mg/6 h of this compound in eight cases of galactosuria [6]. Compounds III:1 and III:3 did not correspond to any known oligosaccharide and furthermore III:3 was absent when galactose was not administered.

These results indicate that certain blood-group-active oligosaccharides are excreted in the urine regardless of nutritional factors and superimposed on these are other oligosaccharides which are probably biosynthesized during galactose transport across the intestinal cells.

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Release of Oligosaccharides from Human Erythrocyte Membranes of Different Blood-Group-P Phenotypes by Trifluoroacetolysis

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Human erythrocyte membranes of different blood-group-P phenotypes have been degraded by trifluoroacetolysis at conditions that will effect transamidation. This degradation resulted in dissolution of the membranes with concomitant degradation of proteins. Oligosaccharide chains linked to proteins and ceramides were released as their pertrifluoroacetylated derivatives, which generally are stable towards further action of the reagent. 2-Acetamido-2-deoxy-sugar residues were converted into their corresponding 2-deoxy-2-trifluoroacetamido derivatives. The mixture of pertrifluoroacetylated oligosaccharides obtained was de-*O*-trifluoroacetylated and de-*N*-trifluoroacetylated, reduced, acetylated, permethylated and analyzed by gas-liquid chromatography/mass spectrometry. Membranes obtained from two individuals with phenotype B, $\bar{p}$ (known to contain an excess of lactosyl ceramide) gave almost identical results with lactose released as the main component. From A₂B, P^k cell membranes small amounts of lactose were obtained but the trisaccharide Gal(α 1-4)Gal(β 1-4)Glc was the major component found. This trisaccharide is most likely derived from the P^k antigen (trihexosyl ceramide). Membranes from a donor with the phenotype B, P₁ yielded small amounts of both lactose and the P^k trisaccharide together with a larger quantity of the tetrasaccharide GalNAc(β 1-3)Gal(α 1-4)Gal(β 1-4)Glc. The tetrasaccharide represents the carbohydrate portion of the P antigen (globoside). From all three types of membranes the B-specific trisaccharide Gal(α 1-3)Gal

| (α 1-2)

Fuc was obtained. This compound is probably derived from specific degradation of blood-group-B-active glycoproteins and/or glycolipids. In addition to the oligosaccharides mentioned above a large number of others were observed but in lower amounts.

Recently a new reaction, trifluoroacetolysis, has been developed which will effect transamidation at conditions where most glycosides and reducing sugars are stable, being converted into their pertrifluoroacetylated derivatives [1–4]. Using this reaction procedures for the isolation of *O*- and *N*-glycosidically linked carbohydrate chains in glycoproteins have been developed [5–7]. It has also been demonstrated that the glycosidic bond to the sphingosine moiety in glycolipids is cleaved specifically by trifluoroacetolysis [8].

Cell surface carbohydrates exist in the form of monosaccharides and oligosaccharides linked either to proteins or lipids which are buried in the cell mem-

brane. The carbohydrate portion of these cell membrane glycoconjugates has been shown to play an important role in several biological functions [9], including cell-cell interactions, receptors for antibodies, enzymes, hormones, viruses, and toxins.

In order to determine the structure and study the biological activity of the carbohydrate portion of glycoconjugates in cell membranes it would be desirable to release the oligosaccharide chains as intact as possible. Such a procedure could then save the more tedious and sometimes impossible task of isolating pure, intact glycoproteins and glycolipids. Trifluoroacetolysis will, as mentioned above, release oligosaccharide chains from both glycoproteins and glycolipids with simultaneous degradation of proteins and lipids and therefore the possibilities for this technique have been investigated using, as models, erythrocyte membranes obtained from individuals of different blood-group-P phenotypes. The glycolipid patterns

Abbreviations. Hex, hexopyranosyl; Gal, D-galactopyranoside; GalOH, galactitol; Glc, D-glucose; GlcOH, glucitol; 6-deHex, 6-deoxyhexopyranosyl; HexOH, hexitol; Fuc, L-fucopyranoside; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranoside; HexNAc, 2-acetamido-2-deoxy-hexopyranosyl; GalNAc, 2-acetamido-2-deoxy-D-galactopyranoside.

of these erythrocyte membranes have been extensively studied [10] and they therefore appeared ideal for a model study.

MATERIALS AND METHODS

Erythrocyte Membranes

Blood samples were obtained from one donor with phenotype B,P₁, two donors (monozygous twins) with phenotype B, $\bar{p}$ and one donor with phenotype A₂B,P₁ $\bar{p}$ at the Blood Banks in Lund, Umeå, and Helsinki, respectively. Membranes were prepared by the method of Dodge et al. [11]. The yield was approximately 1 g per 100 ml of blood.

Oligosaccharides and Glycolipids

Urinary B-active trisaccharide was obtained as previously described [12]. Isomaltotetraose was a gift from Dr Kirsti Granath (Uppsala, Sweden). Trihexosyl ceramide and globoside were gifts from Professor Lars Svennerholm (Gothenburg, Sweden).

Analytical Methods

Sugar analysis was performed after hydrolysis with 90% aqueous formic acid at 100°C for 5 h followed by hydrolysis with 0.25 M aqueous sulphuric acid at 100°C for 18 h, by gas-liquid chromatography [13] and mass spectrometry [14]. Methylation analysis was performed as previously described [15]. Gas-liquid chromatography was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on (a) an SE-30 W.C.O.T. glass capillary column (25 m $\times$ 0.25 mm) at 200°C for partially methylated alditol acetates and at 180–330°C for permethylated oligosaccharide alditols, (b) a glass column (2 m $\times$ 0.5 cm) packed with 3% ECNSS-M on Chromosorb Q at 200°C for alditol acetates. Gas-liquid chromatography/mass spectrometry was performed on a Varian MAT 311A instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionization current of 3 mA and an ion-source temperature of 120°C. The spectra were processed on an on-line computer system (Spectroscopy 100, Varian MAT).

Trifluoroacetylolysis of Erythrocyte Membranes

2.0 g of freeze-dried erythrocyte membranes together with 1.0 mg of isomaltotetraose was treated with trifluoroacetic acid/trifluoroacetic acid anhydride (1:1, v/v) (100 ml) and the reaction mixture was heated at 100°C for 48 h in a sealed glass vessel. (Caution! The mixture is corrosive and under pressure!) After cooling the reaction mixture was concentrated to dry-

Table 1. The P blood group system (after Race and Sanger [17])

Phenotype	Antigens on red cells	Antibodies in serum	Approximate frequency
P ₁	P ₁ , P	—	75%
P ₂	P	anti-P ₁	25%
P ₁ $\bar{p}$	P ₁ , P ^k	anti-P	very rare
P ₂ $\bar{p}$	P ^k	anti-P	very rare
$\bar{p}$	—	anti-PP ₁ P ^k	very rare

Table 2. P^k, P and P₁ glycosphingolipids isolated from human erythrocyte membranes

Specificity	Structure
P ^k	Gal(α 1-4)Gal(β 1-4)GlcCer
P	GalNAc(β 1-3)Gal(α 1-4)Gal(β 1-4)GlcCer
P ₁	Gal(α 1-4)Gal(β 1-4)GlcNAc(β 1-3)Gal(β 1-4)GlcCer

ness, dissolved in methanol (100 ml) and concentrated to dryness again. The resulting product was dissolved in glacial acetic acid (50 ml) and after that distilled water (50 ml) was added. After 1 h at room temperature the solution was evaporated to dryness. Diethyl ether (250 ml) and after that distilled water (150 ml) was added and the mixture was shaken. The water phase was kept and the ether phase was washed three times with water (100 ml each time). The combined water phases were washed three times with diethyl ether (100 ml each time) and evaporated to dryness. The resulting residue was reduced (NaBH₄), acetylated (acetic anhydride/pyridine), permethylated and analyzed by gas-liquid chromatography/mass spectrometry [16].

Trifluoroacetylolysis of Trihexosyl Ceramide and Globoside

The glycolipid (1.00 mg) together with isomaltotetraose (0.50 mg), as stable internal standard, was treated with trifluoroacetic acid/trifluoroacetic acid anhydride (1:1, v/v) (2 ml) at 100°C for 48 h in a sealed glass tube. The reaction mixture was then processed as described above and analyzed by gas-liquid chromatography/mass spectrometry.

RESULTS AND DISCUSSION

The human P-blood-group system consists of three antigens, P₁, P and P^k, and owing to the presence of these antigens in different combination or their absence, five phenotypes can be distinguished (Table 1) [17]. These antigens were first demonstrated on erythrocytes [18] and later on skin fibroblasts and lymphocytes [19]. The chemical nature of the antigens has been elucidated [20–21] (Table 2). Individuals with

the $\bar{p}$ phenotype are lacking all blood-group-P antigens, i.e. trihexosyl ceramide, globoside and P_1 -active glycolipid, but accumulate lactosyl ceramide and gangliosides in their erythrocyte membranes. Lactosyl ceramide can be considered as a precursor substance in the P system [10]. Individuals with the P_1^k phenotype accumulate trihexosyl ceramide and are lacking globoside and P_1 -active glycolipid. The membranes of P_1^k phenotype also accumulate trihexosyl ceramide and are lacking globoside but do contain the P_1 -active glycolipid [10, 22]. It has also been demonstrated that fibroblasts of $\bar{p}$ and P_1^k phenotypes are lacking α -galactosyl and β -N-acetylgalactosaminyl transferase activities, respectively [23].

When the erythrocyte membranes are anhydride with trifluoroacetic acid/trifluoroacetic acid anhydride (1:1, 100°C) they dissolved almost completely in the reagent within 5 h forming a black solution. After 48 h the reaction was stopped and the reaction mixture was de-O-trifluoroacetylated by treatment with methanol and 50% aqueous acetic acid. The resulting mixture contained oligosaccharides originally present in the erythrocyte membranes or those which had been released from glycolipids [8] and/or glycoproteins [5–7]. If an oligosaccharide structure originally contained an acetamido function, this function is transamidated into a trifluoroacetamido group during the trifluoroacetylation reaction [1]. By this transamidation reaction proteins or the protein part of glycoproteins are degraded. In order to reconstitute the transamidated acetamido functions, the oligosaccharides were de-N-trifluoroacetylated by treatment with sodium borodeuteride [1], a reagent that also converted the reducing end of the oligosaccharides into an alditol residue with a deuterium label at C-1. The oligosaccharide mixture was then O-acetylated and N-acetylated, de-O-acetylated and permethylated. The resulting mixture of permethylated oligosaccharide alditols was then analyzed by gas-liquid chromatography/mass spectrometry. Typical multiple ion-tracing chromatograms for the permethylated oligosaccharide alditols obtained from erythrocyte membranes of blood group phenotypes B, P_1 , B, $\bar{p}$ and A $_2$ B, P_1^k are shown in Fig. 1A–C.

Peak a (Fig. 1) which was present in all chromatograms gave a mass spectrum typical for a permethylated Hex(1-4)[1- 2 H]HexOH [14] and had a retention time identical to that of permethylated dGal-(β 1-4)d[1- 2 H]GlcOH. This component is likely to be derived from lactosyl ceramide by acid-catalyzed elimination of the oligosaccharide from the ceramide portion during trifluoroacetylation [8]. The amounts of lactose released from the different erythrocyte membranes (Table 3) are in agreement with previously reported values [10] for the amounts of lactosyl ceramide present in these types of erythrocyte membranes.

Table 3. Amounts of oligosaccharides released from erythrocyte membranes from individuals with different blood group P phenotypes. Results are expressed as mg/g of erythrocyte membranes. (–) Not detected

Phenotype	Release of			
	Gal(β 1-4)-Glc	Gal(α 1-4)-Gal(β 1-4)-Glc	GalNAc-(β 1-3)Gal-(α 1-4)Gal-(β 1-4)Glc	Gal(α 1-3)Gal (α 1-2) Fuc
	mg/g membrane			
B, P_1	1.1	1.2	10	1.3
B, $\bar{p}$	5.5	–	–	1.7
A $_2$ B, P_1^k	–	31	–	2.1

Peak b was also present in all types of investigated erythrocyte membranes and its mass spectrum (Fig. 2) is typical for a permethylated Hex(1-3)[1- 2 H]HexOH.
| (1-2)
6-deHex

Its retention time was identical to that of Gal(α 1-3)[1- 2 H]GalOH. The origin of this oligosaccharide (1-2)
Fuc

charide is less certain but it is only obtained from erythrocyte membranes with blood-group-B activity (unpublished results from the Department of Clinical Chemistry, University Hospital, Lund). It may have been released from blood-group-B-active glycoproteins and/or glycolipids present in the erythrocyte membranes [25, 26]. Soluble blood group substances obtained from ovarian cysts with blood-group-B-activity release the same B-active trisaccharide on trifluoroacetylation (M. A. Chester and S. Svensson, unpublished results) by specific degradation reactions. During trifluoroacetylation the branched GlcNAc residues (Fig. 3) in the type I and type II chains will only have O-trifluoroacetyl groups protecting their glycosidic bonds in the 6-position. This protection is not enough [4] and when the glycosidic bonds of the GlcNAc residues are solvolyzed these residues will be further degraded [2] with release of fucose and the trisaccharide Gal(α 1-3)Gal as their pertrifluoroacetylated derivative (1-2)
Fuc
tives. This trisaccharide is then further partly degraded [3].

Peak c (Fig. 1A and C) gave a mass spectrum (Fig. 4) in which the A and J series of fragments and the fragments obtained by α -cleavage in the alditol chain show that the component in peak c is a permethylated Hex-Hex(1-4)[1- 2 H]HexOH [16]. The mass spectrum and retention time on gas-liquid chromatography were identical with those of an authentic sample of Gal-(α 1-4)Gal(β 1-4)Glc as its permethylated [1- 2 H]alditol

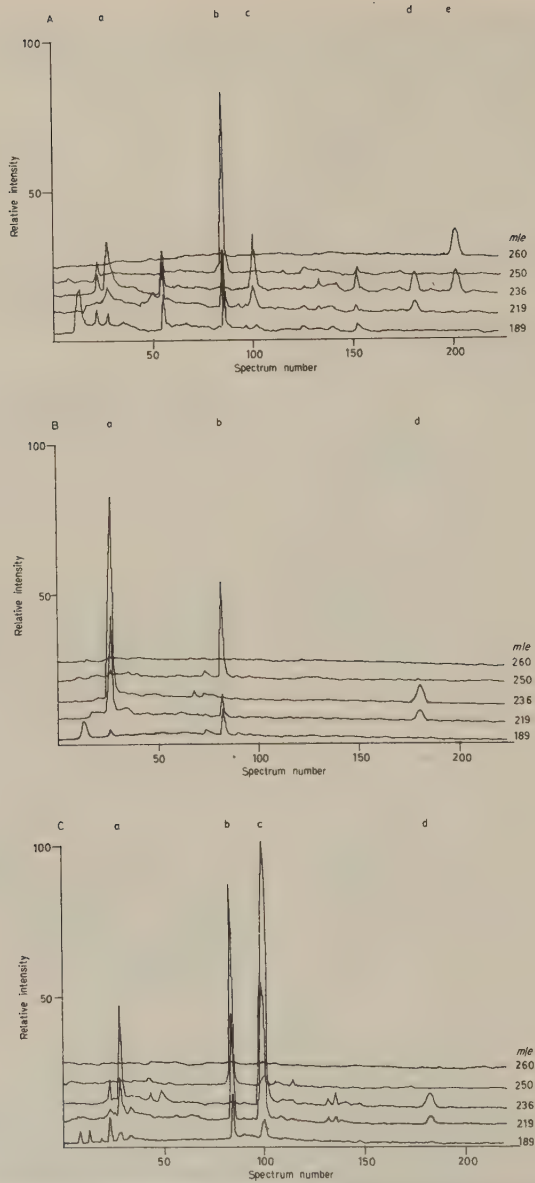


Fig. 1. Multiple ion tracing of gas chromatographic/mass spectrometric analysis of permethylated alditol mixtures obtained from erythrocyte membranes of blood group phenotypes B.P₁ (A), B.p̄ (B), and A₂B.P₁ (C). Peak d identified as the internal standard (isomaltotetraose)

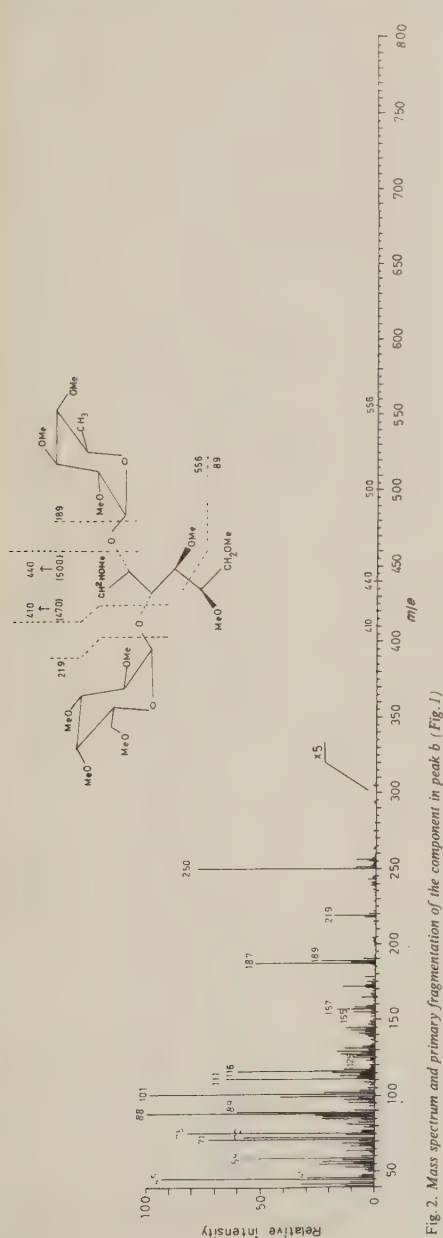


Fig. 2. Mass spectrum and primary fragmentation of the component in peak b (Fig. 1)

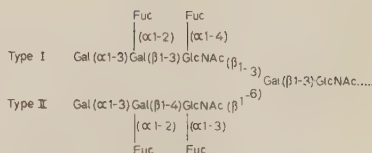


Fig. 3. Suggested composite structure for the outer portion of a B-specific megalosaccharide in soluble blood group substances [24]

derivative which was obtained from trihexosyl ceramide (see Materials and Methods). The identity of the trisaccharide is further corroborated by the fact that it constitutes the carbohydrate portion of the dominating glycolipid (trihexosyl ceramide) in the erythrocyte membranes of P^k cells and that this glycolipid is absent in cells of the p phenotype [10].

Peak e (Fig. 1A) gave a mass spectrum (Fig. 5) which is expected from permethylated HexNAc(1-3)-Hex-Hex(1-4)HexOH by analysis of the A and J series of fragmentation and by inspection of the fragments obtained from α -cleavage of the alditol residue. The mass spectrum and retention time by gas-liquid chromatography were identical with those of a permethylated [1-²H]alditol derivative of authentic GalNAc-(β 1-3)Gal(α 1-4)Gal(β 1-4)Glc. This oligosaccharide represents the carbohydrate portion of globoside and was only obtained from the cells of the P₁ phenotype, as expected.

The oligosaccharide Gal(α 1-4)Gal(β 1-4)GlcNAc-(β 1-3)Gal(β 1-4)Glc which is obtained by trifluoroacetylation, de-N-trifluoroacetylation and de-O-trifluoroacetylation, and re-N-acetylation of the P₁-active glycolipid is too large to be analyzed by gas-liquid chromatography/mass spectrometry and is therefore not accounted for in the above analyses.

In the present study a new technique, trifluoroacetylation is presented by which oligosaccharides can be released intact from glycolipids and glycoproteins. Certain highly branched carbohydrate structures are specifically degraded into smaller units due to lack of protection, by O-trifluoroacetyl groups, of the glycosidic bond. If this technique for release of oligosaccharides is combined with gas-liquid chromatography/mass spectrometry methodology [16] for analysis, it is a powerful technique for analysis of carbohydrate determinants of cell membranes. The method is rapid and sensitive (as little as picogram quantities of oligosaccharides can be analyzed using multiple ion selection techniques).

Thanks are due to Prof. Harry R. Nevanlinna, Helsinki, Finland, for supplying red cells phenotype P₁. The authors are also indebted to Miss Gunilla Lundberg for excellent technical assistance. This work was supported by grants from the Swedish Medical Research Council (O3X-2 and O3X-4956), the Medical Faculty, University of Lund, and Magnus Bergvall's Foundation.

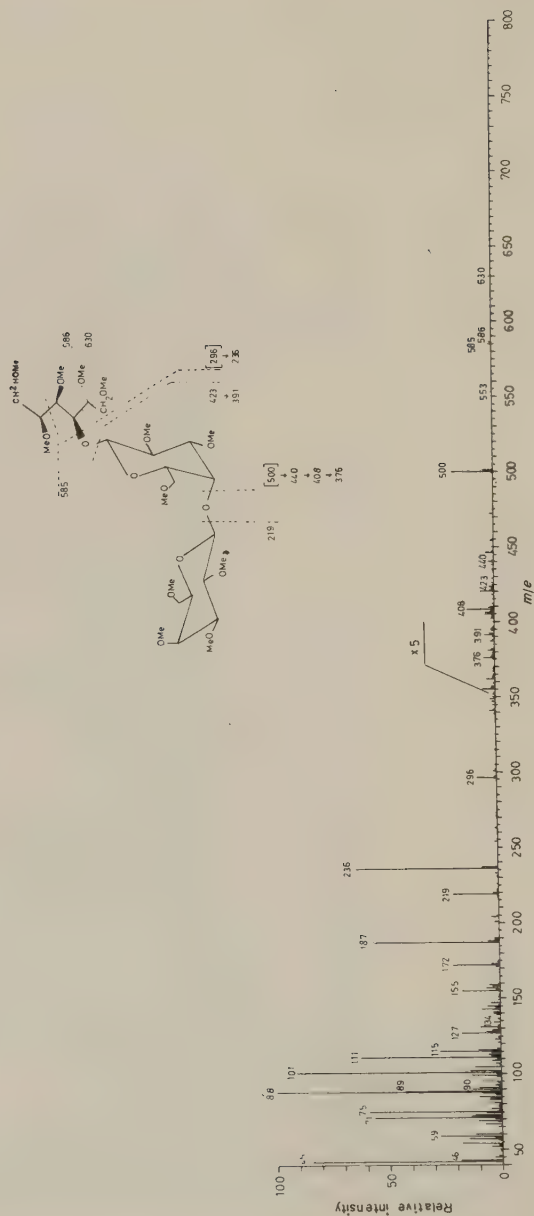


Fig. 4. Mass spectrum and primary fragmentation of the component in peak c (Fig. 1)

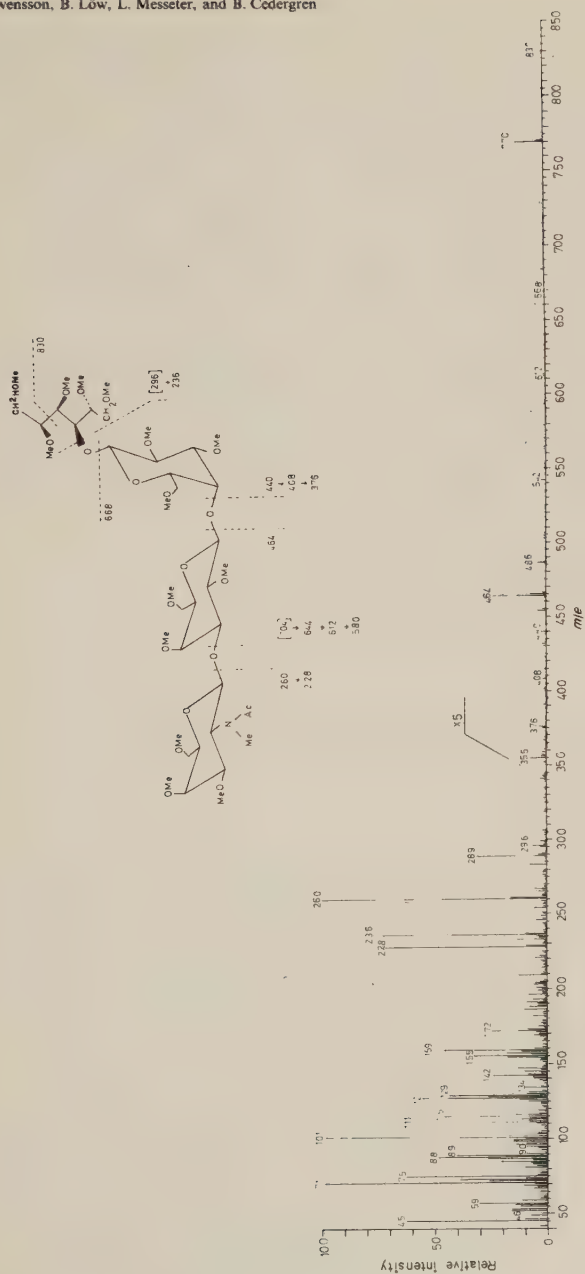


Fig. 5. Mass spectrum and primary fragmentation of the component in peak e (Fig. 1)

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MOUSE MONOCLONAL ANTIBODIES WITH ANTI-A, ANTI-B AND
ANTI-A,B SPECIFICITY; SUPERIOR TO HUMAN POLYCLONAL
ABO REAGENTS

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Running title: Monoclonal ABO antibodies

ABSTRACT

A series of fusions using mouse myeloma cells and spleen cells from mice immunized with blood group substances or human red cells was performed with the aim of obtaining ABO antibodies suitable for routine blood grouping. Eight strongly agglutinating antibodies against antigens in the ABO system were obtained from 18 fusions. These antibodies were tested extensively in manual and automated routines and seven of them were found to be as good as or better than commercial testsera of human origin.

INTRODUCTION

The expectation that monoclonal antibodies will be able to replace conventional polyclonal antibodies in routine ABO grouping has been questioned (1). Recently several attempts to produce this type of antibody were reported (2-6), in which tissue culture cells from human colon carcinoma (2), whole red cells (4, 6), and native (3) and synthetic (5) blood group active glycoproteins were used as immunogens using various immunization procedures. Some of the antibodies were claimed to have satisfactory quality for clinical use (7), but in most cases, however, the titre was low and the reactions with weak subgroups were inadequate.

In the present study, whole red cells, red cell membranes or soluble blood group substances prepared from individuals with blood group A₁, A₂, B or A₂B were used as immunogens, and a high dose booster protocol (8) was adopted in most of the experiments. A large number of mouse-mouse hybridomas secreted antibodies with anti-A, anti-B and anti-A,B specificity and several of these antibodies were shown to be superior to human polyclonal testsera, both in manual and in automated blood grouping.

MATERIALS AND METHODS

Mice

Female mice of the inbred Balb/cABom strain were obtained from G1 Bomholtgaard, Ry, Denmark. The mice were five to nine weeks old when first primed with antigen. They were kept on a standard pellet diet and water ad lib.

Antigens

Purified human ovarian cyst fluid glycoproteins from blood group A₁, A₂ and B individuals were gifts from Drs W.M. Watkins and E.A. Kabat. Red cells from normal blood donors of blood group A₁, A₂ and A₂B were used, either as 10% (v/v) suspensions in saline or as membranes (9) suspended (1%, w/v) in saline.

Immunization procedure

Immunizations were performed according to the protocol in table I.

Table I. Immunization and fusion schedule

Day	Final high-dose booster (substances)	Immunization with cells	Primary response (cells or substances)
	1.	2.	3.
1	50 µg + CFA SC	0.5 ml IP	0.5 ml/50 µg IP
5	-		FUSION
15	50 µg + IFA IP	0.5 ml IP	
19		FUSION	
29	50 µg IP		
30	400 µg IP		
31	400 µg IP		
32	400 µg IP		
33	FUSION		

Abbreviations: CFA Complete Freund's adjuvant (Difco, Detroit, Mich. USA)
 IFA Incomplete Freund's adjuvant (Difco)
 SC Subcutaneous injection
 IP Intraperitoneal injection

A final high-dose booster schedule (8) was usually followed when soluble blood group substances were used as antigens. With red cells, a shorter schedule with two consecutive intraperitoneal injections without adjuvant was mostly adopted. A few experiments were performed using a single injection of antigen with fusion during the primary response.

Hybridoma techniques

Cell fusion, cloning and cultivation of hybridoma cell lines were performed according to standard procedures (10-12). Myeloma cell lines SP2/0, P3-NS1 Ag-4, or P3x63 Ag8-653.1 were used. Ascitic fluid was produced in Pristane^R (Aldrich, Beerse, Belgium)-treated mice.

Selection of antibodies and preparation of reagents

The culture medium from growing hybridomas was tested for haem-agglutinating antibodies in microtitre plates (Linbro, Hamden Conn, USA) with red cells of different ABO groups, and in ELISA (13), using microtitre plates (Microelisa^R Immulon, Dynatech GMBH, Plochingen, W Germany) coated with blood group active glycoproteins. Ascitic fluids and cell culture medium from expanded cultures were centrifuged to remove cellular debris. Cell culture supernatants were decolourized by passage through a charcoal column. Sodium chloride (see also results), and sodium azide (0.1%) were added. Sodium was determined by flame photometry and osmolality by freezing point depression. The solutions were sterilized by filtration and stored at 4°C.

Isotype determination

The antibody isotype was determined by double immunodiffusion (14) using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM antisera, obtained from Miles, Elkhart, Ind, USA.

Agglutination tests

Manual tests were performed using undiluted culture medium or ascitic fluid diluted in 0.9% NaCl and 5% red cell suspensions which were incubated at 22°C, either on tiles for 20 min or in tubes for 1 h. Reactions were read against a well-lit background, and positive reactions were graded from (+) to 4+. Red cell samples from healthy blood donors, newborn infants (cord samples) and patients were tested. In titration studies, twofold serial dilutions with saline were performed. Tube techniques with 1 h incubation were used.

Avidity testing was performed both on slides with 40 - 50% red cell suspensions in homologous serum and on tiles with 5% red cell suspensions in saline. Agglutination strength was recorded after 30, 60, 90 and 120 sec. Specificity testing was performed using a large panel (35 donors) of extensively phenotyped cells. Standard two-step papain and bromelain methods, and saline testing were used in tube techniques with incubation at 4, 22 and 37°C. The reactions were read microscopically. For comparison, 20 commercial polyclonal test sera of human origin (subsequently called polyclonal sera) were also analyzed.

Table II. Compiled results.

Fusion number	Antigen	Immunization schedule*	Myeloma cell line	Total number of hybridomas	Hybridomas positive in haemaggl.	Haemaggl. in specificity	Designation of useful haemaggl. clones
1.1	A ₁ substance	1	NS1	92	2	Anti-A(A ₁)	1.1.1
1.2	"	3	NS1	118	0		
1.3	"	1	653	380	8	Anti-A(A ₁)	
1.4	"	1	653	82	3	Anti-A	1.4.1
1.5	"	1	SP2/0	73	1	Unknown	
1.6	"	1	653	-**	1	Anti-A(A ₁)	
2.1	A ₂ substance	1	NS1	37	0		
2.2	"	1	SP2/0	100	0		
2.3	"	1	SP2/0	432	29	Anti-A	2.3.2
3.1	B substance	1	653	84	25	Anti-A(A ₁)	
4.1	A ₁ membranes	3	NS1	0	6	Anti-B	3.1.1
4.2	"	3	NS1	14	0		
5.1	A ₂ red cells	2	653	160	21	Anti-A(A ₁)	
5.2	"	2	653	192	2	Anti-A, B	5.1.2
5.3	"	2	SP2/0	384	1	Unknown	
6.1	A ₂ B red cells	2	653	387	10	Anti-N	5.3.1
6.2	"	2	653	175	1	Anti-B	6.1.1
7.1	A ₁ +A ₂ red cells	3	SP2/0	10	12	Anti-A, B	6.1.5
					-**	Unknown	

* See Table I.

** Lost in tissue culture

Automated testing was performed with the Groupamatic 360 system using antibody diluted in saline and bromelinated cells in a standard procedure (15). Helix pomatia extract (Biotest, Frankfurt, Germany) was used for automated A-grouping. In addition to the automated evaluation, a visual check was also performed on all reactions.

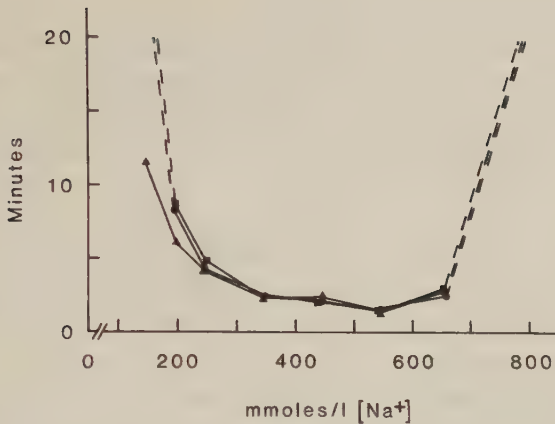
RESULTS

The data from 18 fusions are compiled in table II. Ten fusions were performed after immunization with blood group active substances and eight using red cells or red cell membranes as antigen. Most fusions gave rise to several hybridomas that produced ABO-specific antibodies, but the majority of these antibodies were not agglutinating or only very weakly so. The specificity was in these cases determined with ELISA and these results will be published elsewhere. Immunization with A or B substances gave rise to anti-A or anti-B antibodies, respectively, whereas red cell immunization also resulted in anti-A,B and anti-N antibodies and several antibodies of hitherto unknown specificity.

It was observed that an increase in sodium chloride concentration increased the antibody avidity as measured by the time to obtain a 4+ reaction. Similar effects were observed with other salts (KCl, CaCl₂, KI, MgCl₂, MgSO₄), but none of these was better than sodium chloride, which was selected as an additive. Examples of the salt effect are given in figure 1, which shows the reactions of anti-B 6.1.1 at various salt concentrations. Without addition of salt, the pH optimum was between 6.5 and 7.5, but when salt was added this pH optimum broadened, and complete reactions were obtained most rapidly if the sodium ion concentration of the antibody reagent was between 350 and 550 mmol/l, giving an osmolality of 700 to 1100 mosm.

Fig 1. Time taken to obtain complete haemagglutination using antibody 6.1.1 (anti-B) and varying NaCl concentrations.

0.2 ml of antibody reagent with the sodium ion concentrations shown (abscissa) was mixed with 0.1 ml of a 5% suspension of B (▲), AB (●) or A₂B (■) red cells in physiological saline. The cell culture supernatant was initially dialyzed against 0.1M sodium phosphate buffer pH 7.5.



A total of seven hybridomas were selected for further study: three that produced anti-A, two anti-B and two anti-A,B. Six hybridomas produced IgM antibodies and one (6.1.5) was an IgG3 antibody. Their specificity was confirmed with ELISA. Both the cell culture supernatant and ascitic fluid produced by these hybridomas were used in this study, but most of the agglutination experiments have been performed with supernatants. The results of the manual agglutination tests are given in tables III-V.

Blood samples from individuals with blood groups A_1 , A_2 , A_1B , B and O were studied (table III).

Table III. Agglutination reactions with red cells of different ABO groups.

	A_1	A_2	A_{cord}	A_1B	B	B_{cord}	O
<u>Anti-A</u>							
1.1.1*	4	(+) to 1	(+) to 4	4	neg	neg	neg
1.4.1	4	4	3 to 4	4	neg	neg	neg
2.3.2	4	4	4	4	neg	neg	neg
Polyclonal I	4	4	4	4	neg	neg	neg
Polyclonal II	4	4	3 to 4	4	neg	neg	neg
<u>Anti-B</u>							
3.1.1	neg	neg	neg	4	4	4	neg
6.1.1	neg	neg	neg	4	4	4	neg
Polyclonal I	neg	neg	neg	4	4	4	neg
Polyclonal II	neg	neg	neg	4	4	4	neg

Table III, cont.

	A ₁	A ₂	A _{cord}	A ₁ B	B	B _{cord}	O
<u>Anti-A,B</u>							
5.1.2**	4	4	4	4	4	4	neg
6.1.5	4	4	4	4	4	4	neg
Polyclonal I	4	4	4	4	4	4	neg
Polyclonal II	4	4	4	4	4	4	neg

* Ascites, diluted 1:250

** Ascites, diluted 1:200

Otherwise the monoclonal reagents were tested as undiluted cell culture supernatants.

All monoclonal reagents gave potent reactions quite comparable to those of the polyclonal sera, except 1.1.1, which had agglutinating properties similar to a Dolichos biflorus extract.

Table IV. Agglutination reactions with erythrocytes from 10 A₂B persons.

		1	2	3	4	5	6	7	8	9	10	Score
<u>Anti-A</u>												
1.4.1		4	4	4	4	3	3	4	3	4	4	35
2.3.2		4	4	4	4	3	4	4	3	3	3	36
Polyclonal I		3	2	2	3	1	3	2	3	2	2	23
"	II	3	3	3	3	2	3	3	3	3	3	29
"	III	3	3	3	3	2	3	3	3	2	2	27
"	IV	3	3	3	3	2	3	2	3	3	3	28
"	V	3	3	3	3	3	3	3	3	3	3	30
"	VI	4	3	3	3	3	3	3	3	3	3	31
<u>Anti-A,B</u>												
6.1.5		4	4	4	4	3	4	4	3	3	3	36
Polyclonal I		4	4	4	4	3	4	4	3	3	3	36
"	II	2	2	3	3	3	4	3	3	3	3	29
"	III	3	3	3	3	3	4	3	3	3	3	31
"	IV	2	3	3	3	3	3	3	3	3	2	28
"	V	3	3	3	3	3	3	3	3	3	3	30
"	VI	3	3	3	3	3	3	3	3	3	3	30

The monoclonal reagents were tested as undiluted cell culture supernatants.

Table IV shows the results of agglutination tests with blood samples from 10 A₂B donors. With red cells of this type, both of the monoclonal anti-A sera and the anti-A,B were clearly superior to the polyclonal sera tested. The reactions with monoclonal and polyclonal anti-B were of equal strength (4+) and are not given in the table.

Table V. Agglutination reactions with weak ABO variants.

	A ₃ B	A _x	A _x	A _m	A _{end}	A _{el}	A _{el}	B ₃	B ₃
<u>Anti-A</u>									
1.1.1	1*	(+)*	(+)*	neg	neg	neg	(+)*	neg	neg
2.3.2	2*	1 *	1 *	neg	neg	neg	(+)*	neg	neg
Polyclonal I	1*	w	neg	neg	neg	neg	neg	neg	neg
" II	(+)*	neg	neg	neg	neg	neg	neg	neg	neg
<u>Anti-B</u>									
3.1.1	4	neg	neg	neg	neg	neg	neg	neg	neg
6.1.1	4	neg	neg	neg	neg	neg	neg	(+)	neg
Polyclonal I	4	neg	neg	neg	neg	neg	neg	neg	neg
" II	4	neg	neg	neg	neg	neg	neg	neg	neg
<u>Anti-A,B</u>									
6.1.5	4	(+)*	neg	(+)*	1*	1*	neg	neg	1*
Polyclonal I	3	2 *	2*	neg	neg	neg	neg	(+)*	(+)*
" II	3	1 *	1 *	neg	neg	neg	neg	neg	neg

* = Mixed field appearance

w = Weak reaction

The monoclonal reagents were tested as undiluted cell culture supernatants.

Table V shows reactions with weak ABO variants. Both monoclonal anti-A reagents gave positive reactions with the two A_x samples after 20 min incubation at 22°C. After incubation for 1 h in tubes, the polyclonal sera also gave (+) to 1+ reactions with the A_x cells, but under these conditions the monoclonal sera gave 1+ to 2+ reactions. Both monoclonal anti-A reagents gave weak reactions with one of the A_{el} samples but did not react with the other. None of the polyclonal reagents reacted with these cells. The only anti-B reagent to react with a B₃ sample (classified according to Race and Sanger (16)) was the monoclonal reagent 6.1.1 after incubation for 1 h at 4°C and centrifugation (500 g, 60 sec). The monoclonal anti-A,B reacted more strongly than the polyclonal sera with the A₃B sample and the A subgroups other than A_x.

The results of automated ABO blood grouping (Groupamatic 360) are given in Table VI. Each batch of samples was first analyzed using the routine reagents and was then retested using the monoclonal antisera. The number of reactions not accepted by the machine due to weak agglutination was of the same order for all reagents.

All the reagents tested were completely specific, except the anti-A,B 5.1.2 which gave weak (+) to 1+) reactions with enzyme-treated group O cells. All other monoclonal reagents were negative with these cells.

In avidity testing with 40 - 50% red cell suspensions, the antibody 2.3.2 was more avid than polyclonal anti-A, particularly with A₂B red cells, and the anti-B antibody 6.1.1 was more avid than polyclonal anti-B, particularly with A₁B red cells. The monoclonal antibodies 1.4.1 and 3.1.1 reacted more slowly than the polyclonal sera, but the time to obtain a 4+ reaction was within the two minute limit set in the European Agreement (17). When tested with 5% red cell suspensions on tiles, the avidity generally was higher with monoclonal than with polyclonal sera.

A monoclonal and a polyclonal antibody of each specificity (A; B; A,B) were tested for ability to agglutinate Balb/c mouse erythrocytes. As can be seen in table VII, none of the monoclonal reagents reacted with the murine cells, whereas the polyclonal anti-B and anti-A,B gave weakly positive reactions.

Table VII. Agglutination reactions with Balb/c mouse red cells.

Reagent	Mouse number				
	1	2	3	4	5
<u>Anti-A</u>					
1.2.1	-	-	-	-	-
Polyclonal I	-	-	-	-	-
<u>Anti-B</u>					
3.1.1	-	-	-	-	-
Polyclonal I	1*	1*	1*	1*	1*
<u>Anti-A,B</u>					
6.1.5	-	-	-	-	-
Polyclonal I	2*	2*	2*	2*	2*

* Mixed field appearance

Monoclonal reagents were undiluted cell culture supernatants.

Table VI. A comparison of automated ABO grouping (Groupamatic 360) using commercial reagents and the monoclonal antisera (cell culture supernatants). The reagents were diluted as indicated.

Blood Group	Accepted results	Weak reactions				Monoclonal reagents				Non-specific reactions
		Routine reagents								
		Anti-A	Anti-B	Anti-A,B		Anti-A	Anti-B	Anti-A,B		
A	5235	7		4		10		5		0
B	1211		3	2			4	4		0
AB	572	16		3		4	3	2		0
O	4461									0
11479		35				32				0

DISCUSSION

The major advantages of monoclonal antibodies in blood grouping compared to conventional polyclonal antibodies are the constant composition of the antibody reagent and the possibility of obtaining large quantities at relatively low cost. The implication of these advantages is that it should be possible to obtain vastly superior reagents for use in blood group serology. Each reagent would consist of a single antibody, or a carefully defined mixture of monoclonal antibodies, with well characterized specificity.

With this aim in mind, a series of fusions was performed using several immunization and fusion protocols and a number of potentially suitable antigens. Although a total of 18 fusions was carried out, the use of three different immunization routes, three different myeloma cell lines and three different types of antigen (table I) precludes the definition of an optimum fusion schedule.

Both cell culture supernatants and ascitic fluids from each hybridoma were studied. The advantage of ascitic fluid antibodies is their high concentration in this fluid which enables them to be used in high dilution. This dilution also has the effect of diluting the mouse's own natural antibodies which have been shown to react with determinants expressed on human fetal and other glycoproteins (18). The concentration of the antibodies from ascitic fluid in this study was too low to allow the required high dilution, possibly due to the in vivo breakdown of the IgM molecules formed by the hybridoma cells (19). The cell culture supernatants were sufficiently potent to be used in routine blood grouping, so were generally preferred in haemagglutination tests.

The specificities of the haemagglutinating antibodies obtained from each fusion using mice immunized with blood group substances was as expected specific for the particular blood group, with the exception of one antibody of unknown specificity from fusion 1.4 and an anti-A(A₁) obtained from fusion 2.3 using blood group A₂ substance. The latter may be explained by the presence of an A₁ domain in the A₂ substance, i.e. a small region in the glycoprotein having a higher than normal density of A determinants.

Immunization with red cells gave a more complex picture of haemagglutinating antibody formation. No fusion using A_2 , A_2B or A_1+A_2 red cells resulted in the formation of an anti-A. Most notable was the formation of 21 anti- $A(A_1)$, two anti-A,B and two antibodies of unknown specificity from a fusion (5.1) using A_2 cells. Also notable was the formation of 10 anti-B, one anti-A,B and 12 unknown but no anti-A from fusion 6.1 using A_2B cells.

The production of an anti-B antibody in mice is of interest since most mouse strains, including Balb/c are considered to have a blood group B-like antigen on their erythrocytes, and thus are expected not to produce an anti-B (3). Mouse red cells were tested with the monoclonal and polyclonal reagents (table VII) and were found to react weakly with the polyclonal anti-B and anti-A,B, but not with the anti-A. None of the corresponding monoclonal reagents reacted with the murine cells, which explains why these antibodies can be produced in mice. The mouse "B-like" antigen is therefore recognized by other specificities in the polyclonal anti-B sera which are absent from the monoclonal reagents.

The addition of salts to the monoclonal antibodies increases the rate of the agglutination reaction, which remains constant over a relatively wide range, but causes dramatically reduced avidity outside certain limits. Isotonic saline (0.15 M) is generally used for blood grouping purposes, but it is well known that the rate of the reaction between polyclonal IgG antibodies and antigen is increased by a reduction of ionic strength from 0.15 M to 0.03 M (20, 21). The reaction between purified polyclonal IgM anti-A and A red cells, on the other hand, is not increased in media of low ionic strength (22). The mechanism of changed association rates in media with different ionic strengths is not known and either antigen or antibody could be affected. The addition of salts might cause conformational changes favouring binding to the antigen combining site.

Manual tests (tables III-V) using 5% red cell suspension indicated that the monoclonal antibodies chosen for study were all as potent and specific as the polyclonal reagents when tested with A_1 , A_2 , A_1B , B and O cells, and A and B cord cells (table III).

The exception was clone 1.1.1 which has the characteristics of an anti-A₁ antibody. Using A₂B cells the monoclonal anti-A and anti-A,B reagents were clearly better than the corresponding polyclonal sera (table IV), whereas all the anti-B reagents gave a complete reaction. More demanding tests using erythrocytes with weaker A or B antigens (table V) indicated that when there were any reactions at all, the monoclonal anti-A and anti-B reagents were slightly better. The reactions of the anti-A,B sera were rather variable.

In automated grouping the performance of the monoclonal reagents was also excellent (table VI). The monoclonal anti-A performed as well as the Helix extract which is normally used in automated grouping since the reactions of polyclonal anti-A reagents are too weak with A₂B cells (15). The weak, apparently non-specific reaction seen with enzyme-treated group O red cells and the monoclonal anti-A,B reagent in specificity testing was not observed in automated grouping of more than 11000 samples in the Groupamatic system in which bromelinated red cells are used.

Using a 40 - 50% red cell suspension, some of the monoclonal reagents were less avid than the polyclonal reagents, but this could easily be remedied by concentrating the monoclonal antibody supernatants five-tenfold. However, this is unnecessary (7) for routine use.

So far, the monoclonal reagents have shown no loss of titre when stored at 4°C for at least one year or at room temperature for over two months.

The present study clearly shows that these monoclonal reagents perform extremely well, both in manual and in automated blood grouping. The reagents were in many instances better than polyclonal sera, particularly with A₂B cells or weaker ABO variants. This was confirmed in a clinical evaluation in which monoclonal reagents were used in parallel with polyclonal sera (unpublished data). In manual grouping of some 2000 samples (mainly cord samples and samples from patients with haematological disorders and a positive direct antiglobulin test) the monoclonal reagents generally gave more clear-cut reactions and firmer agglutinates. The monoclonal reagents were in no instance inferior to the polyclonal sera, nor were any non-specific reactions observed.

With the production of a battery of different monoclonal antibodies it is possible to mix two or more such reagents to obtain an "oligoclonal" reagent with well-defined properties. Thus in the future it might be possible to compose anti-A and anti-B reagents that can recognize and distinguish all ABO variants, thus making anti-A,B reagents redundant.

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IMMUNOCHEMICAL CHARACTERIZATION OF A MONOCLONAL
ANTI-Le^b BLOOD GROUPING REAGENT

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ABSTRACT

A haemagglutinating monoclonal anti-Le^{bL} antibody has been obtained from a mouse-mouse hybridoma after immunization with the Le^b-active oligosaccharide lacto-N-difucohexaose I, coupled to edestin. Of 13 other antibodies obtained, one was able to agglutinate enzyme-treated erythrocytes of all ABO groups and three more were considered to be anti-Le^{bH} haemagglutinins. The remaining nine antibodies did not agglutinate red cells. The specificity of the stronger of the anti-Le^{bL} haemagglutinins was examined by studying its binding to a number of glycoconjugates and oligosaccharides.

INTRODUCTION

Anti-Le^b haemagglutinins appear occasionally as antibodies in human sera of Le(a-b-) and Le(a+b-) individuals (1). They have been produced in goats by immunization with Le^b-active soluble blood group substances (2) or synthetic glycoconjugates containing Le^b-active oligosaccharides coupled to protein carriers (3), and in mice as monoclonal antibodies derived from animals immunized with human tumour cells (4). Most anti-Le^b haemagglutinins react weakly with test cells and exhibit anti-Le^{bH} activity, i.e., they agglutinate cells from O Le(a-b+) and A₂ Le(a-b+) individuals but fail to react with cells of other ABO phenotypes. Anti-Le^{bL} sera, which react almost equally well with Le(a-b+) cells of all ABO blood groups, are more useful for Le^b blood grouping but they are rare reagents. In this report we describe an anti-Le^{bL} mouse monoclonal IgM antibody produced by a hybridoma derived from a mouse immunized with a glycoconjugate containing the Le^b-active milk oligosaccharide, lacto-N-difucohexaose I (LND I), coupled to edestin (5). This antibody reacts strongly with all Le(a-b+) erythrocytes and it is available as a uniform Le^b blood grouping reagent in virtually limitless supply.

EXPERIMENTAL PROCEDURE

Materials

Glycoconjugates containing an average of 23 moles of the Le^b-active milk oligosaccharide, lacto-N-difucohexaose I (LND I), per mole of edestin or 20 moles of LND I per mole of bovine serum albumin (BSA) were prepared as previously described (5,6). Purified human ovarian cyst glycoprotein from a B, Le^b individual was a gift from Dr W.M. Watkins.

Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Difco Laboratories, Detroit, MI, USA. Anti-mouse immunoglobulin-horseradish peroxidase was obtained from Dako, Copenhagen, Denmark. 5-Amino salicylate (5-AS) (Sigma, St. Louis, Mo., USA) was purified as previously described (7).

Immunizations

Antigen was prepared from a stock solution of LND I-edestin in 0.05 M NaOH stored at -10°C . Just prior to injection aliquots were neutralized with one equivalent of HCl, diluted in buffered saline to a final antigen concentration of 1 mg/ml and administered subcutaneously or emulsified first with an equal volume of CFA or IFA. The immunization schedule is given in Table I.

Table I. Immunization route.

Day after primary injection	Antigen μg	Adjuvant
0	50	CFA
108	50	IFA
141	100	IFA
231	50	-
232	400	-
233	400	-
234	400	-
235	FUSION PERFORMED	

Hybridomas

Spleen cells from three female Balb/cABom mice (Gl Bomholtgård, Ltd, Ry, Denmark) immunized with LND I-edestin as described above were fused at a ratio of 10/1 with Sp 2/0 cells in 50% polyethylene glycol as described by Kennett (8). Cloning was performed as described by Nowinski et al (9) using Balb/cABom thymocytes as feeder cells. Hybridomas whose supernatant fluids contained immunoglobulins that bound LND I-BSA and B,Le^b substance in ELISA and agglutinated human Le(a-b+) erythrocytes were cloned and grown as ascites tumours in Balb/cABom mice.

Table II. Structures of oligosaccharides

Trivial name	Structure
1. H-disaccharide	Fuc α 1-2Gal
2. 2'-Fucosyllactose	Fuc α 1-2Gal β 1-4Glc
3. 3-Fucosyllactose	Gal β 1-4Glc 3 1 Fuc α
4. Lactodifucotetraose	Gal β 1-4Glc 2 3 1 1 Fuc α Fuc α
5. Lacto- <u>N</u> -tetraose	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc
6. Lacto- <u>N</u> -fucopentaose I	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 2 1 Fuc α
7. Lacto- <u>N</u> -fucopentaose II	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 4 1 Fuc α
8. Lacto- <u>N</u> - <u>neo</u> -fucopentaose II	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc 3 1 Fuc α
9. Lacto- <u>N</u> -difucohexaose I	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 2 4 1 1 Fuc α Fuc α
10. Lacto- <u>N</u> -difucohexaitol I	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glucitol 2 4 1 1 Fuc α Fuc α
11. Lacto- <u>N</u> -difucohexaose II	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 4 3 1 1 Fuc α Fuc α

Table II, cont

12. Lacto- <u>N</u> - <u>neo</u> -difucohexaose I	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc 2 3 1 1 Fuca Fuca
13. Lacto- <u>N</u> -trifucoheptaose II	Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc 2 4 2 1 1 1 Fuca Fuca Fuca
14. Lacto- <u>N</u> - <u>neo</u> -trifucoheptaose II	Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc 2 3 2 1 1 1 Fuca Fuca Fuca
15. VI _H	Gal β 1-3GlcNAc β 1-4Gal 2 4 6 1 1 1 Fuca Fuca Fuca
16. A-trisaccharide	GalNAc α 1-3Gal 2 1 Fuca
17. B-trisaccharide	Gal α 1-3Gal 2 1 Fuca
18. A-pentasaccharide	GalNAc α 1-3Gal β 1-4Glc 2 3 1 1 Fuca Fuca
19. B-pentasaccharide	Gal α 1-3Gal β 1-4Glc 2 3 1 1 Fuca Fuca
20. Fucosylgalactosylinositol	Fuc α 1-2Gal β 1-0-inositol
21. Difucosylgalactosylinositol	Gal β 1-0-inositol 2 0 1 1 Fuca Fuca

Oligosaccharides

Oligosaccharides were isolated from human urine as previously described (10). Oligosaccharide VI_H (11) was a gift from Dr G Strecker. (³H)LND I-alditol was prepared by reduction of LND I with tritium labelled sodium borohydride (12) (specific activity = 15.6 Ci/mmol, New England Nuclear Corp., Boston, MA, USA). Oligosaccharide structures are given in Table II.

Glycolipids

The glycolipids used are listed in table III. Glycolipids I, II and V were isolated from human small intestine (13-15) and glycolipids III and IV from dog small intestine (14,15). Glycolipid V has been shown to react with the "Siedler" antibody (unpublished experiments performed by C.A. Tilley, University of Toronto) which is thought to be an anti-A₁Le^b antibody (16).

Table III. Glycolipids used for ELISA assays.

	Structure	Blood group activity
I	Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-1Cer 4 1 Fuca	Le ^a
II	Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-1Cer 2 4 1 1 Fuca Fuca	Le ^b
III	Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1Cer 3 1 Fuca	-
IV	Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1Cer 2 3 1 1 Fuca Fuca	-
V	GalNAcα1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-1Cer 2 4 1 1 Fuca Fuca	-

ELISA

Screening of antibody production was carried out using solid phase ELISA (17) with LND I-BSA and B, Le^b-substance (1 μg/well) as target antigens bound to microtitre plates (Dynatech Imulon^R, Plochingen, W. Germany).

In specificity studies the binding of antibody to glycolipid was assayed by adding 20 μ l of glycolipid in methanol to the wells of the microtitre plate. When the methanol had evaporated (about 30 min) 200 μ l of phosphate buffered saline pH 7.3 (PBS) containing 1% BSA was added and the plates were left at room temperature for 2 h (4). After emptying the wells, 20 μ l of antibody (undiluted cell culture supernatant or serial dilutions made in fresh cell culture medium) and 20 μ l of 1% BSA in PBS was added. The plates were covered with parafilm, shaken gently for 30 min, then left overnight at room temperature. After emptying the plates and washing the wells four times with 200 μ l of 1% PBS, 50 μ l of anti-mouse immunoglobulin-horseradish peroxidase (diluted 1:500 with 1% BSA in PBS) was added to each well. Incubation for 30 min at 37°C was followed by washing as above with 1% BSA in PBS and addition of 100 μ l substrate (1 g recrystallized 5-aminosalicylate in 1 l of 0.01 M sodium phosphate buffer pH 6.0) at room temperature. The reaction was followed at 450 nm using a microtitre plate scanner (LKB multiskan).

For inhibition studies 10 ng of Le^b-glycolipid were used per well, 20 μ l of an aqueous solution of inhibitor and 20 μ l of 1% BSA in PBS were added, and 20 μ l of undiluted cell culture supernatant was used as the antibody. Otherwise the ELISA procedure was as described above.

Radioimmunoassay

Antibody specificity was also characterized by comparing various oligosaccharides as inhibitors of binding of (³H) LND I-alditol using a nitrocellulose filter assay as previously described (3) except that incubations were carried out for 16 h at 4°C.

Haemagglutination

Haemagglutination studies were performed in tubes by mixing 50 μ l of undiluted cell culture supernatant with 50 μ l of a 2 - 5% erythrocyte suspension (washed three times and suspended in 0.15 M sodium chloride) with or without the addition of 50 μ l papain solution (18). After incubation at 4°C for 30 min the tubes were centrifuged at 500x g for 20 sec, then shaken gently and the reactions were read macroscopically against a well lit background.

Isotype determination

The antibody isotype was determined by double immunodiffusion (19) using rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM antisera, obtained from Miles, Elkhart, Ind., USA.

RESULTS AND DISCUSSION

Detection of anti-Le^b hybridomas

From two separate fusion experiments (768 wells plated) a total of 14 hybridomas producing antibodies reacting with LND I-BSA were detected by ELISA screening. Eight of these produced IgM antibodies, all of which bound B,Le^b substance.

Haemagglutinating antibodies

The haemagglutinating properties of these eight hybridoma culture supernatant fluids were examined using O,Le(a-b-) and O,Le(a+b-) erythrocytes and Le(a-b+) cells from donors belonging to various ABO blood groups. Five antibodies gave some positive reactions, (Table IV) but the remaining three hybridoma antibodies had no haem-agglutinating activity.

Table IV. Haemagglutination by hybridoma cell culture supernatants.

Erythrocyte phenotype									
		Le(a-b+)					Le(a+b-)	Le(a-b-)	
Hybridoma		A ₁	A ₂	A ₁ B	A ₂ B	B	O	O	O
9.1	S	-	-	-	-	-	-	-	-
	P	-	w	-	-	w	+	-	-
9.2	S	-	-	-	-	-	w	-	-
	P	w	+	w	+	+	+	-	-
9.3	S	-	-	-	-	-	w	-	-
	P	-	-	-	-	-	+	-	-
10.1	S	-	-	-	-	-	-	-	-
	P	-	w	-	-	-	w	-	-
10.2	S	+	+	+	+	+	+	-	-
	P	+	+	+	+	+	+	-	-

Agglutination is graded: - = absent, w = weak, + = strong

S = saline suspended cells

P = papain treated cells

None of the hybridoma antibodies agglutinated O Le(a-b-) and O Le(a+b-) cells either before or after treatment with papain, as expected. Three hybridoma antibodies (9.1, 9.3 and 10.1) reacted with papainized cells of blood group O Le(a-b+) but only weakly or not at all with Le(a-b+) cells of other ABO blood groups.

Hybridoma antibody 9.2 reacted with papain treated Le(a-b+) cells of all ABO blood groups. Hybridoma antibody 10.2 gave good reactions with Le(a-b+) cells of all ABO blood groups tested, even without papain treatment. When compared with commercially available anti-Le^b antisera, antibody 10.2 was consistently superior to the human anti-Le^b reagents in tests on 80 blood samples from Le(a-b+) donors of different ABO groups. The monoclonal antibody (10.2) was as good as or better than commercially available goat anti-Le^b antisera. Thus, of eight anti-Le^b hybridomas selected after immunization with LND I-edestin, three produced antibodies exhibiting weak Le^{bH} activity and two exhibiting anti-Le^{bL} activity. The stronger of the two anti-Le^{bL} antibodies (10.2) was studied further.

Specificity of hybridoma antibody 10.2

The activities of various oligosaccharides as inhibitors of binding of (³H) LND I-ol to hybridoma antibody 10.2 in a nitrocellulose filter radioimmunoassay are shown in Figure 1.

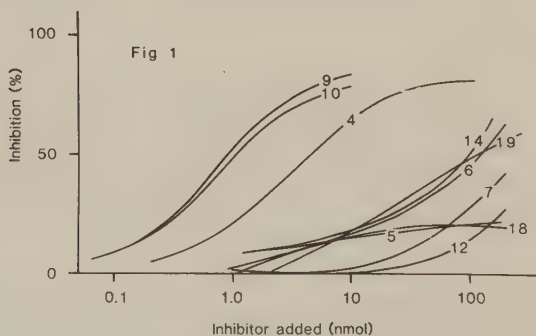


Fig. 1.
Inhibition of binding of (³H) lacto-N-difucohexaitol I to hybridoma antibody 10.2 by oligosaccharides. See Table II for oligosaccharide structures.

Lacto-N-difucohexaose I and lacto-N-difucohexaitol I are the most potent inhibitors and are equally active. Lactodifucotetraose is about one eighth as active as lacto-N-difucohexaose I on a molar basis and the remaining seven structurally analogous oligosaccharides are less than 0.1% as active.

The patterns of inhibition for hybridoma antibody 10.2 and for a polyclonal anti- Le^b serum from a goat immunized with LND I-polylysine (3) differ very little except that lactodifucotetraose is a much better inhibitor of 10.2.

Le^b antigens on human erythrocytes are glycolipids (20) absorbed to the plasma membrane from circulating lipoproteins (21).

Figure 2 shows the binding of undiluted hybridoma antibody 10.2 to various amounts of an Le^b -active glycolipid (glycolipid II, Table III) consisting of the oligosaccharide lacto-N-difucohexaose I linked to ceramide. Maximum binding was obtained with about 8 ng of glycolipid or more.

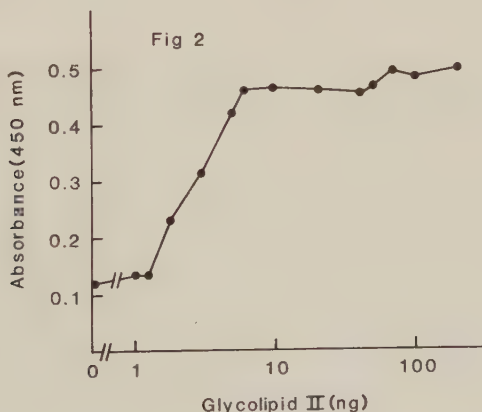


Fig. 2.
Binding of antibody in tissue culture supernatant from hybridoma 10.2 to microtitre plates coated with Le^b -active glycolipid measured using the ELISA technique.

Further characterization of the specificity of antibody 10.2 was performed by examining the ability of the oligosaccharides given in Table II to inhibit the binding of the antibody to the Le^b glycolipid using an ELISA technique. Four oligosaccharides inhibited binding, as shown in Figure 4.

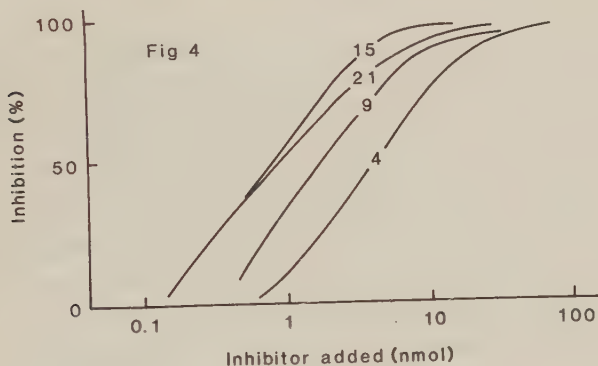


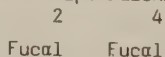
Fig. 4. Inhibition of binding of hybridoma antibody 10.2 to the Le^b-active glycolipid using the ELISA technique. The oligosaccharides are numbered as shown in Table II.

The oligosaccharides containing the Le^b structure (LND I and VI_H) were the most potent inhibitors together with difucosylgalactosylinositol. Lactodifucotetraose, a closely related structure, was also an effective inhibitor, but consistently less potent than the other three. All the other oligosaccharides gave no inhibition of binding in concentrations at which the above four oligosaccharides gave essentially complete inhibition. Similar results were obtained using microtitre plates coated with 10 ng/well of LND I-BSA.

Radioimmunoassay gave results very similar to those obtained by ELISA, with 50% inhibition of binding by lacto-N-difucohexaose I and lactodifucotetraose being obtained at inhibitor concentrations of 3, 7 and 10 μ M respectively.

The above results demonstrate that monoclonal antibody 10.2 is an IgM antibody with haemagglutinating properties comparable to those of the best currently available commercial anti-Le^b reagents. The reagent can be characterized as an anti-Le^{bL} on the basis of its reaction with erythrocytes of all ABO groups. Its lack of anti-Le^a activity was also confirmed in binding studies using purified glycolipids and oligosaccharides.

Antibody 10.2 was unable to bind to glycolipid IV (Table III), nor was it inhibited by oligosaccharide 12 (Table II). However, the milk oligosaccharide lactodifucotetraose, which has a closely related oligosaccharide structure, was a potent inhibitor of anti-Le^b activity. A possible explanation is the presence of glucose in the oligosaccharide of the N-acetylglucosamine of the glycolipid. The major conformational difference between the Le^b-active tetrasaccharide structure (based on the type I chain) and the analogous structure based on the type II chain (c.f. glycolipids II and IV in Table III) is an inversion of the N-acetylglucosamine ring (22), with transposition of the bulky N-acetyl group to a position which apparently prevents binding. As lactodifucotetraose contains no N-acetyl group, it might assume a conformation sufficiently similar to the Le^b-active sequence Gal β 1-3GlcNAc β 1-



to permit complementary fit with the antigen combining site of antibody 10.2.

The monoclonal antibody 10.2 is in many ways similar to anti-Le^b antibodies obtained from mice immunized with a human adenocarcinoma cell line (4) but additionally has haemagglutinating properties and a more extensively defined specificity.

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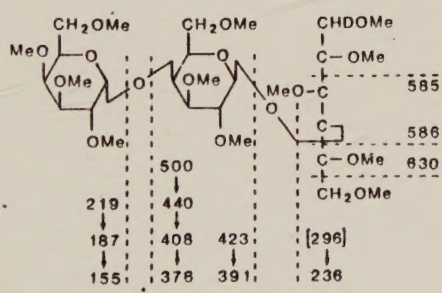
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ERRATA

p. 29	column 1 line 1	tetra-en penta	should read tetra-and penta
p. 30	" 2 "	4 D-fucose	" <u>L</u> -fucose
p. 30	" 2 "	12 4,6,-di-O-methyl-galactose	" 4,6,-di-O-methyl-galactitol
p. 43	table 3	Gal(α1-3)Gal (α1-2) Fuc	Gal(α1-3)Gal (α1-2) Fuc
p. 43	column 2 "	3 Hex(1-3)(1- ² H)HexOH (1-2) 6-deHex	Hex(1-3)(1- ² H)HexOH (1-2) 6-deHex
p. 43	" "	6 Gal(α1-3)(1- ² H)GalOH (α1-2) Fuc	Gal(α1-3)(1- ² H)GalOH (α1-2) Fuc
p. 43	" "	26 Gal(α1-3)Gal (α1-2) Fuc	Gal(α1-3)Gal (α1-2) Fuc

p. 46 figure 4 The structure should be replaced by the following:



p. 47 figure 5 The structure should be replaced by the following:

